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 [Ala³¹,Alb³²]NPY and the Y1/Y5 receptor-binding peptide [Leu³¹,Pro³²]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 [³²P]NPY with high affinity, a low affinity displacement of [³²P]NPY was observed at the Y1 and the Y5 receptors. Consequently, competition at the Y2 receptor occurred with a considerably lower Ki 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) 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 (Y₁, Y₂, and Y₅), which all belong to the large family of G-protein-coupled receptors (GPCR). They are coupled to G proteins and accordingly inhibit adenylosuccinate (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 Y₁ 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-
tional and selective NPY displacer by displacing NPY from its receptors Y1, Y2, and Y5.

EXPERIMENTAL PROCEDURES

Materials—The N\(^{-}\)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; 2,5,7,8-tetramethylbenzothiazole-6-sulfonil for Arg. The 4-(2'-4',6'-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\(^{-}\)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 \(\times\) 40 min). The Fmoc protection 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; HOBr and DIC were dissolved in DMF (0.5 μM). One hour after addition of this solution to the pre-swollen resin, 5 eq of N-ethylidiso- propanolamine (DIEPA) were added. The coupling reaction was stopped after 15 h by washing the resin with DMF, methanol, ethylene chloride, DMF, and diethyl ether (each 1 ml at 1 ml each). The peptides were cleaved from the resins with a mixture of trifluoroacetic acid/thianniso/ 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/v). Characterization of the peptides was achieved by mass spectrometry and analytical high pressure liquid chromatography.

Y2 Receptor Mimetic 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'-TCTAATAAGCAGCTA- CTAATAAGGAAAGGGAACCTGAG-40N-AGAAAGGAAGAGGACGCGTACGATGCACTG-3', 5'-PCR primer Mic-(1-39), 5'-TCTAAATACGACTCA- TCTAATAAGGAAAGGGAACCTGAG-40N-AGAAAGGAAGAGGACGCGTACGATGCACTG-3' (underlines indicate the T7 promoter and boldface indicates the HindIII 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 10 mM) for 10-12 h at 37 °C in 40 mM Tris/HCl buffer, pH 8, containing 12 mM MgCl\(_2\), 5 mM dithio-}

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 10 mM) for 10-12 h at 37 °C in 40 mM Tris/HCl buffer, pH 8, containing 12 mM MgCl\(_2\), 5 mM dithio-}

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 10 mM) for 10-12 h at 37 °C in 40 mM Tris/HCl buffer, pH 8, containing 12 mM MgCl\(_2\), 5 mM dithio-

Equilibrium Dissociation Constants—Binding studies were performed with 1.5 mM 5'-32P-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\(_2\)PO\(_4\), 5 mM Na\(_2\)HPO\(_4\), 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 monophos-}

(Y2R) = (P + R + Kd) - (P + R + Kd)² - 4PR\(^{1/2}\) (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 agaroase 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.

In vitro Selection 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% confluency 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% Bactracin), and counted. After centrifugation (800 rpm for 5 min) the pellet was resuspended in the incubation buffer and diluted to 2 \(\times\) 10\(^6\) cells/ml. 200 μl of cell suspension (5 \(\times\) 10\(^6\) cells) were incubated simultaneously with 25 μl of oligonucleotide solution in increasing concentrations and a constant amount of \(^{32}\)P-hypotyrosyl-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 \(^{32}\)P-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 GraphPad Prism 3.02 using the “sigmoidal dose response (variable slope)” Equation 2.

\[
Y = \left(\frac{\text{resp}_{\text{Bottom}} + \text{resp}_{\text{Top}} - \text{resp}_{\text{Bot}}}{1 + 10^{\log IC_{50} - x \text{Hill}}}ight) \times \left(1 + 10^{\log IC_{50} - x \text{Hill}}ight)^{-1}
\]

where Y indicates response, whereas Y starts at bottom (resp_{Bottom}) and goes to top (resp_{Top}) with sigmoidal shape; x indicates log of concentration of aptamer. Equation 2 is also designated as the “four parameter logistic equation.”

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

\[
K_d = IC_{50}(1 + L \times K_d)^{-1}
\]

where L is the concentration of competed \(^{32}\)P-NPY which was 1 mM for Y1 and 2 mM 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 nucleo-}

As the C-terminal pentapeptide of NPY has recently been described (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 terminal (Fig. 1A). From an RNA pool with a complexity of 9 \(\times\) 10\(^{14}\) different sequences 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 terminal (Fig. 1A). From an RNA pool with a complexity of 9 \(\times\) 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
amplification (Fig. 1). Fig. 1B 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.

**Fig. 1.** The selected target NPY and its binding to the enriched library from cycle 12. A, sequence of the N-terminally bio- tinylated 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.

**Fig. 2.** Sequences of 2-amino pyrimidine 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.
Y2 Receptor Mimetic Anti-NPY Aptamers

TABLE I

| Aptamer clone | NPY | ScNPY | 18–36 | 36–13 | Ahx5–24 | hPP |
|---------------|-----|-------|-------|-------|---------|-----|
| DP3           | 0.37| NA    | 1.16  | 0.76  | 0.71    | NA  |
| DP4           | 0.41| NA    | NA    | NA    | NA      | NA  |
| DP11          | 0.47| NA    | NA    | NA    | NA      | NA  |
| Pool          | NA  | NA    | NA    | NA    | NA      | NA  |

$^a$ Biotinylated full-length NPY.
$^b$ Sequence of the scrambled NPY, SKPQRDNAPAYLHRLYNLTPRYYNH2.
$^c$ Amino acids 1–17 were deleted.
$^d$ Amino acids 1–12 were deleted.
$^e$ Amino acid residues 5–24 were substituted by an aminohexanoic acid spacer.
$^f$ NA, no affinity.

TABLE II

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

| Entry | Name       | Peptide sequence                  | Binding |
|-------|------------|-----------------------------------|---------|
| 1     | NPY        | Biotin-YPSPDNPGEDAPARLYSYLHINLTYRQY-NH2 | ++      |
| 2     | 1–10       | Biotin-YPSPDNPGEDAPARLYSYLHINLTYRQY-NH2 | –       |
| 3     | 5–20       | Biotin-YPSPDNPGEDAPARLYSYLHINLTYRQYNH2 | –       |
| 4     | 25–34      | Biotin-RHYINLINLTYRQY-NH2           | –       |
| 5     | 25–36      | Biotin-RHYINLINLTYRQY-NH2           | –       |
| 6     | 18–28      | Biotin-ARYSALHY1-NH2               | –       |
| 7     | 18–32      | Biotin-ARYSALHYINLTYRQY-NH2        | –       |
| 8     | Ahx24      | Biotin-YPSP---RHYINLINLTYRQY-NH2   | ++      |
| 9     | 13–36      | Biotin-PAEDLARYSYLHINLTYRQY-NH2   | ++      |
| 10    | 18–36      | Biotin-ARYSALHYINLTYRQY-NH2        | ++      |
| 11    | [Ala]$^{a}$18–36 | Biotin-ARYSALHYINLTYRQY-NH2 | ++      |
| 12    | [Ala]$^{a}$18–36 | Biotin-ARYSALHYINLTYRQY-NH2 | ++      |
| 13    | [Ala]$^{a}$18–36 | Biotin-ARYSALHYINLTYRQY-NH2 | ++      |
| 14    | [Ala]$^{a}$18–36 | Biotin-ARYSALHYINLTYRQY-NH2 | ++      |
| 15    | [Leu]$^{a}$, [Pro]$^{a}$ | Biotin-YPSPDNPGEDAPARLYSYLHINLTRPYYNH2 | –       |
| 16    | [Ala]$^{a}$, [Ala]$^{a}$ | Biotin-YPSPDNPGEDAPARLYSYLHINLIA5RQY-YNH2 | +       |

$^a$ Biotin-RPPDGDNAPAYLHRLYNLTPRYYNH2.

In constructs 1–7, 9, and 10 the remaining amino acid residues of several N- and C-terminally truncated porcine NPY variants were 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).

Table I shows the $K_d$ 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.

Table II shows the binding behavior of the aptamer to all tested NPY constructs as reflected in a competitive situation in which the aptamer competes with various receptor subtypes for NPY binding. We used 3H-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 shows monophasic competition (Table III). In contrast, the competition curves at the Y1 and Y5 receptors exhibit a completely different shape (Fig. 3). 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 $K_d$ values (Table III). Thus, the aptamer-dependant inhibition of NPY/Y2 receptor interaction occurs with an $K_d$ 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
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$^{25}$ (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-termially 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...
Obviously the aptamer stabilizes a similar conformation of NPY as the Y2 receptor (Fig. 4A). At a DP3 concentration of about 200 nM [3H]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. 4B). 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-idiotype 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 $K_i$ 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.

Acknowledgments—We thank Günter Mayer and Michael Blind for helpful discussions and Ernst-Ludwig Winnacker for continuous support. The technical support of Hella Späte is kindly acknowledged.

REFERENCES

1. Larhammar, D. (1996) Regul. Pept. 65, 165–174
2. Cerdà-Reverter, J. M., and Larhammar, D. (2000) Biochem. Cell Biol. 78, 371–392
3. Glover, I. D., Barlow, D. J., Pitts, J. E., Wood, S. P., Tickle, I. J., Blundell, T. L., Tatamoto, K., Kimmel, J. R., Wellmer, A., Strassburger, W., and Zhang, Y. S. (1984) Eur. J. Biochem. 142, 379–385
4. Conlon, J. M., Bjornholm, B., Jorgensen, F. S., Youson, J. H., and Schwartz, T. W. (1991) Eur. J. Biochem. 199, 283–298
5. Chronwall, B. M., DiMaggio, D. A., Massari, V. J., Pickel, V. M., Ruggiero,
Y2 Receptor Mimetic Anti-NPY Aptamers

D. A., and O'Donohue, T. L. (1985) Neuroscience 15, 1159–1181
6. Morley, J. E., Hernandez, E. N., and Flood, J. F. (1987) Am. J. Physiol. 253, E516–E522
7. Loftus, T. M., Jaworsky, D. E., Frehywot, G. L., Townsend, C. A., Ronnett, G. V., Lane, M. D., and Kuhajda, F. P. (2000) Science 288, 2379–2381
8. Flood, J. F., Hernandez, E. N., and Morley, J. E. (1987) Brain Res. 421, 289–290
9. Heilig, M., and Murison, R. (1987) Regul. Pept. 19, 221–231
10. Albers, H. E., Ferris, C. F., Leeman, S. E., and Goldman, B. D. (1984) Science 223, 833–835
11. Hall, A. C., Earle-Cruikshanks, G., and Harrington, M. E. (1999) Eur. J. Neurosci. 11, 3424–3432
12. Calza, L., Giardino, L., Zanni, M., Velardo, A., Parchi, P., and Marrama, P. (1990) Regul. Pept. 27, 127–137
13. Bischoff, A., and Michel, M. C. (1999) Trends Pharmacol. Sci. 20, 104–106
14. Ingenhoven, N., and Beck-Sickinger, A. G. (1999) Curr. Med. Chem. 6, 1055–1066
15. Palczewski, K., Kumasaka, T., Hori, T., Behlke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
16. Sautel, M., Rudolf, K., Wittneben, H., Herzog, H., Martinez, R., Munoz, M., Eberlein, W., Engel, W., Walker, P., and Beck-Sickinger, A. G. (1996) Mol. Pharmacol. 50, 285–292
17. Beck-Sickinger, A. G., Wieland, H. A., Wittneben, H., Willim, K. D., Rudolf, K., and Jung, G. (1994) Eur. J. Biochem. 225, 947–958
18. Cabrele, C., Langer, M., Bader, R., Wieland, H. A., Doods, H. N., Zerbe, O., and Beck-Sickinger, A. G. (2000) J. Biol. Chem. 275, 36043–36048
19. Cabrele, C., and Beck-Sickinger, A. G. (2000) J. Pept. Sci. 6, 97–122
20. Rudolf, K., Eberlein, W., Engel, W., Wieland, H. A., Willim, K. D., Entzeroth, M., Wiesen, W., Beck-Sickinger, A. G., and Doods, H. N. (1994) Eur. J. Pharmacol. 271, R11–R13
21. Soll, R. M., Dingér, M. C., Lundell, I., Larhammer, D., and Beck-Sickinger, A. G. (2001) Eur. J. Biochem. 268, 2828–2837
22. Elling, C. E., Nielsen, S. M., and Schwartz, T. W. (1995) Nature 374, 74–77
23. Wieland, H. A., Eckard, C. P., Doods, H. N., and Beck-Sickinger, A. G. (1998) Eur. J. Biochem. 255, 595–603
24. Klug, S. J., Hutenhofer, A., Kroenmer, M., and Famulok, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6676–6681
25. Famulok, M. (1994) J. Am. Chem. Soc. 116, 1698–1706
26. Jellinek, D., Green, L. S., Bell, C., and Janjic, N. (1994) Biochemistry 33, 10450–10456
27. Eckard, C. P., Cabrele, C., Wieland, H. A., and Beck-Sickinger, A. G. (2001) Molecules 6, 448–467
28. Rist, B., Wieland, H. A., Willim, K. D., and Beck-Sickinger, A. G. (1995) J. Pept. Sci. 1, 341–348
29. Cheng, Y., and Prussoff, W. H. (1973) Biochem. Pharmacol. 22, 3099–3108
30. Gold, L., Polsky, B., Uhlenbeck, O., and Yarus, M. (1995) Annu. Rev. Biochem. 64, 763–797
31. Wilson, D. S., and Szostak, J. W. (1999) Annu. Rev. Biochem. 68, 611–647
32. Famulok, M., and Mayer, G. (1999) Curr. Topics Microbiol. Immunol. 243, 123–136
33. Famulok, M., Mayer, G., and Blind, M. (2000) Acc. Chem. Res. 33, 591–599
34. Pagratis, N. C., Bell, C., Chang, Y.-F., Jennings, S., Fitzwater, T., Jellinek, D., and Dang, C. (1997) Nat. Biotechnol. 15, 68–73
35. Lee, S. W., and Sullenger, B. A. (1997) Nat. Biotechnol. 15, 41–45
36. Buckman, J., Green, L. S., Beeson, J., Waugh, S., Gillette, W. L., Henninger, D. D., Claesson-Welsh, L., and Janjie, N. (1998) J. Biol. Chem. 273, 20556–20567
37. Green, L. S., Jellinek, D., Bell, C., Beebe, L. A., Feistner, B. D., Gill, S. C., Jucker, F. M., and Janjie, N. (1995) Chem. Biol. 2, 683–695
38. Jellinek, D., Green, L. S., Bell, C., Lynott, C. K., Gill, N., Vargesse, C., Kirschreiter, G., McGe, D. P., Absinghe, P., Picken, W. A., Shapiro, R., Rikfkin, D. B., Moscatelli, D., and Janjie, N. (1995) Biochemistry 34, 11383–11392
39. Eaton, B. E., and Picken, W. A. (1995) Annu. Rev. Biochem. 64, 837–863
40. Heidenreich, O., Picken, W., and Eckstein, F. (1995) FASEB J. 7, 90–96
41. Bard, J. A., Walker, M. W., Branchek, T. A., and Weinsbank, R. L. (1995) J. Biol. Chem. 270, 26762–26765
42. Lundell, I., Blenqvist, A. G., Berglund, M. M., Schober, D. A., Johnsen, D., Statnick, M. A., Gadski, R. A., Gehlert, D. R., and Larhammar, D. (1995) J. Biol. Chem. 270, 29123–29128
43. Xu, W., and Ellington, A. D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7475–7480
44. Niewlandt, D., Wecker, M., and Gold, L. (1995) Biochemistry 34, 5651–5659
45. Williams, K. P., Liu, X. H., Schumacher, T. N., Lin, H. Y., Ausiello, D. A., Kim, P. S., and Bartel, D. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11285–11290
46. Blind, M., Kolanus, W., and Famulok, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3606–3610
47. Beck-Sickinger, A. G., Koppen, H., Hoffmann, E., Gaida, W., and Jung, G. (1993) J. Recept. Res. 13, 215–228
48. Ingenhoven, N., and Beck-Sickinger, A. G. (1997) J. Recept. Signal. Transduct. Res. 17, 407–418
49. Ingenhoven, N., Ecker, C. P., Gehlert, D. R., and Beck-Sickinger, A. G. (1999) Biochemistry 38, 6897–6902
50. Moser, C., Bernhardt, G., Michel, J., Schwarz, H., and Busehauer, A. (2000) Clin. J. Physiol. Pharmacol. 78, 134–142