Ala[31]-Aib[32]: Identification of the Key Motif for High Affinity and Selectivity of Neuropeptide Y at the Y_5-Receptor[†]

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ABSTRACT: The turn-inducing sequence Ala-Aib introduced into positions 31 and 32 of neuropeptide Y (NPY) and its analogues has been identified as the key structure for Y_5-receptor selectivity. Analogues of NPY and PP/NPY chimera containing the motif Ala-Aib were prepared; these peptides turned out to be selective for the Y_5-receptor. The affinity of the NPY-based peptides was in the range of 6−150 nM, while the affinity of three (Ala-Aib)-containing PP/NPY chimera was in the range of 0.2−0.9 nM. The circular dichroism spectra of the Aib analogues in aqueous solution were all characteristic of an α helix; however, they had different intensities of the two negative bands at 220 and 208 nm. Affinity and selectivity for the Y_5-receptor were correlated with the ratio of the ellipticity at 220 nm versus the one at 208 nm (*R*), which indicates the presence of a pronounced helix ($R > 1$) versus a less stabile one ($R < 1$). When *R* was in the range 0.74−0.96, the affinity at the Y_5-receptor was in the range >5 nM, while there was complete loss of affinity at the Y_4-receptor. $R > 1.15$ was associated with very high affinity at the Y_5-receptor and weak affinity at the Y_4-receptor. These results suggest that the selectivity of the Ala[31]-Aib[32] motif for the Y_5-receptor derives from a specific conformation that must be correlated with the bioactive conformation of NPY at this subtype.

Neuropeptide Y (NPY)[1] (*1*) is a 36-amino acid peptide amide and is the most conserved member of a hormone family, the so-called NPY family, which further contains the pancreatic polypeptide (PP) and the peptide YY (PYY). NPY is widely distributed within the central and peripheral nervous systems and exerts several physiological functions, such as vasoconstriction, anxiolysis, regulation of neurotransmitter release, increase in memory retention, and stimulation of food intake. NPY and related peptides activate at least four different receptor subtypes that belong to the large family of the G-protein coupled receptors and are designated by a capital Y: Y_1, Y_2, Y_4, and Y_5 (*2*). A fifth receptor has been found in mice, but only an inactivated protein is expressed in humans (*3*). There is strong evidence that the Y_1- and Y_5-receptor subtypes mediate the NPY-induced increase in food intake (*4*). So far, feeding studies have been carried out in vivo by administration of NPY analogues which are Y_1- or Y_5-receptor preferring ligands, but they are not highly specific relative to the other subtypes (*5*, *6*); for example, NPY (2−36) has been shown to be equally potent at the receptors Y_2 and Y_5 and still activates the Y_1-receptor. The shorter analogues of NPY, (3−36) and (13−36), prefer the Y_2- and Y_5-receptor subtypes, but some Y_1-receptor activity is also maintained. [Leu[31], Pro[34]]-NPY and [Pro[34]]-PYY are equipotent at the receptors Y_1, Y_4, and Y_5. Only [D-Trp[32]]-NPY has been shown selectively to activate the Y_5-receptor; however, it is a weak agonist (*5*) and, additionally, we found that it also binds to the Y_2-receptor with nanomolar affinity (*7*). All of these analogues have shown orexigenic properties (*5*, *6*). Because of the lack of specificity, these peptides are not very suitable tools to distinguish the individual role of the Y_1- and Y_5-receptor subtypes in the stimulation of food intake induced by NPY agonists.

A further approach to the in vivo characterization of the feeding receptors has been based on the application of antagonists. BIBP 3226 (*8*) and BIBO 3304 (*9*) are potent and highly selective ligands at the Y_1-receptor, and their administration inhibited the NPY-stimulated increase in food intake. However, it has been observed that the injection of BIBP 3226 is associated with abnormal behavior, and this has suggested that the compound might inhibit NPY-induced feeding via a nonspecific mechanism (*10*). Additionally, Haynes et al. have reported that BIBP 3226 failed to inhibit the response to rat NPY in rats (*11*). 1229U91, an antagonist at the Y_1-receptor (*12*), was successful in inhibiting the NPY-induced increase in food intake (*11*, *13*, *14*); however, it should be taken into account that 1229U91 acts as an agonist at the Y_4-receptor (*15*). Recently, inhibition of food intake

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[1] Abbreviations: Ahx, 6-aminohexanoic acid; Aib, aminoisobutyric acid; CD, circular dichroism; DIC, *N*,*N*′-diisopropylcarbodiimide; FMOC/*t*-Bu, fluorenylmethoxycarbonyl/*tert*-butyl; Hyp, hydroxyproline; NPY, neuropeptide Y (p = porcine, c = chicken); PP, pancreatic polypeptide (a = avian, h = human); PYY, peptide YY.

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has been observed after administration of the Y_5-receptor-selective antagonist CGP 71683A (*16*). Again, the results obtained by using NPY antagonists at the receptors Y_1 and Y_5 cannot clarify the role of NPY and its receptors in feeding behavior.

In this work, we present a new class of NPY analogues and PP/NPY chimera which selectively bind to the Y_5-receptor. We have based the development of these ligands on our knowledge of structure−activity relationships of mutants of NPY and their receptors. The specificity for the Y_5-receptor subtype relative to the receptors Y_1, Y_2, and Y_4 has been obtained by the incorporation of the dipeptide Ala-Xxx at positions 31−32, where Xxx is Aib, Pro, or hydroxyproline (Hyp). Gross-structural features of the unligated peptides in aqueous solution were evaluated by CD spectroscopy. The biological relevance of the most promising candidates was tested through competition assays utilizing ^{3}H-labeled NPY in combination with cell lines that selectively express one of the Y-receptor subtypes. These selective analogues will be very useful tools to characterize this receptor in vivo.

EXPERIMENTAL PROCEDURES

Materials. The N^{α}-Fmoc-protected amino acids were purchased from Alexis (Läufelfingen, Switzerland). Side-chain protecting groups were *tert*-butyl for Asp, Glu, Ser, Thr, and Tyr; Boc for Lys; trityl for Asn, Gln, and His; and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg. The 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy (Rink Amide) and the 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamidonorleucylaminomethyl (Rink Amide AM) resins were obtained from Novabiochem (Läufelfingen, Switzerland). *N*-Hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA), thioanisole, *p*-thiocresol, 1,2-ethanedithiol, trimethylbromosilane, piperidine, 1-methyl-2-pyrrolidinone, *tert*-butyl alcohol, dimethylformamide (DMF) (puriss.), sodium hydrogenphosphate, and potassium dihydrogenphosphate were obtained from Fluka (Buchs, Switzerland). *N*,*N*′-Diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland). DMF (pure) and diethyl ether were purchased from Scharlau (La Jota, Barcelona, Spain). Acetonitrile was obtained from Romil (Cambridge, England). 50% Dulbecco's modified eagle medium/50% Ham's F12, minimum essential medium with Earl's salts and phosphate-buffered saline (PBS) were purchased from Gibco (Life Technologies, Basel, Switzerland). Fetal calf serum, glutamine, and nonessential amino acids were obtained from Boehringer Mannheim (Germany). Geneticin, bacitracin, and bovine serum albumin were purchased from Sigma (Buchs, Switzerland). ^{3}H-Propionyl-NPY was purchased from Amersham (Cleveland, OH).

Peptide Synthesis. The peptides were synthesized by solid-phase technique on an automated multiple-peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) using Rink Amide and Rink Amide AM resins (30 mg, resin loading 0.45−0.51 mmol/g). Peptide chain assembly was performed by using Fmoc-strategy and a double coupling procedure with 10-fold excess Fmoc-amino acid, HOBt, and DIC in DMF (2 × 40 min). The Fmoc-deprotection step was accomplished by 40% piperidine in DMF for 3 min, 20% piperidine for 7 min, and finally 40% piperidine for 5 min. The removal of the amino acid side-chain protecting groups and the peptide cleavage from the resin were accomplished in one step by using the cleavage mixture TFA/thioanisole/thiocresol (90/5/5 v/v) for 3 h. For the cleavage of the Met-containing peptides ethanedithiol was added as scavenger (2−4% v/v). The fully deprotected peptides were precipitated from ice-cold diethyl ether. The suspensions were centrifuged at 5 °C, the ether was decanted, and then the peptides were suspended again in fresh ether and centrifuged. The washings with cold ether were repeated 4 times. Finally, the peptides were dissolved in *tert*-butyl alcohol/water (3/1 w/w) and lyophilized. The peptides containing methionine sulfoxide were reduced after lyophilization by using the reduction mixture TFA/ethanedithiol/trimethylbromosilane (96/2.4/1.6 v/v) for 30−40 min, under a N_2 atmosphere within the first 5 min, then in tightly closed centrifuge tubes (*17*). The reduced peptides were recovered from ice-cold ether, washed as described previously, purified to homogenity, and finally lyophilized.

The peptides were characterized by electrospray ionization mass spectrometry (SSQ 710, Finnigan MAT, Bremen, Germany) and by analytical reversed-phase HPLC on a LiChrospher RP-18 column (5 μm, 3 × 125 mm, Merck, Darmstadt, Germany) using 0.08% TFA in acetonitrile (A) and 0.1% TFA in water (B) as eluting system (20−70% A over 35 min at the flow rate of 0.6 mL/min).

Circular Dichroism. The CD spectra were recorded using a JASCO model J720 spectropolarimeter over 250−180 nm at 20 °C in a N_2 atmosphere. The CD spectra were performed on peptide solutions at the concentration range of 30−40 μM. Each measurement was repeated 4 times using a thermostatable sample cell with a path of 0.02 cm and the following parameters: response time of 2 s, scan speed of 20 nm/min, sensitivity of 10 mdeg, step resolution of 0.2 nm, and bandwidth of 2 nm. The CD spectrum of the solvent was subtracted from the CD spectra of the peptide solutions to eliminate the interference from cell, solvent, and optical equipment. High-frequency noise was reduced by means of a low-path Fourier transform filter. The ellipticity was expressed as the mean-residue molar ellipticity $[\Theta]_R$ in deg cm^2 dmol^{-1}.

Cell Culture. BHK cells transfected with hY_1-, hY_2-, hY_4-, rY_5-, or hY_5-receptors were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 5% penstrep, and 0.1% Geneticin (*9*). SMS−KAN cells (hY_2-receptor) were grown in 50% Dulbecco's modified Eagle medium/50% nutrient mix Ham's F12 with 15% fetal calf serum, 4 mM glutamine, and 1% nonessential amino acids (*18*). Cells were grown to confluency at 37 °C and 5% CO_2.

Receptor Binding Assays. Cells were resuspended in incubation buffer (minimum essential medium with Earl's salts containing 0.1% bacitracin, 50 μM Pefabloc SC, and 1% bovine serum albumin). A total of 200 μL of the suspension containing ca. 600 000 cells were incubated with 25 μL of a 10 nM solution of ^{3}H-propionyl-NPY (^{125}I-PP for the Y_4-receptor) and 25 μL of NPY or analogue at different concentrations. Nonspecific binding was defined in the presence of 1 μM cold NPY. After 1.5 h at room temperature, the incubation was terminated by centrifugation at 2000*g* and 4 °C for 5 min. The pellets were then washed once with PBS by centrifugation, resuspended in PBS, and mixed with the scintillation cocktail. Radioactivity was determined by using a β-counter. Experiments were repeated

Table 1: Amino Acid Sequence and Analytical and Biological Data of the NPY Analogues and of the PP/NPY Chimera[a]

No.	Peptide	Amino acid sequence	M.W.$_{exp}$ [amu]	M.W.$_{calc}$ [amu]	Binding affinity (IC_{50} [nM]) Y_1	Y_2	Y_4	Y_5
1	[Ala^{31}, Aib^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**AB**RQRY	4195.0	4195.7	1 000	760	1 000	5.9
2	[Ala^{31}, Aib^{32}, Pro^{34}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**AB**RPRY	4162.8	4164.6	> 400	> 1 000.	260	16.4
3	[Ala^{29}, Aib^{30}, Ala^{31}, Aib^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYI**ABAB**RQRY	4125.5	4124.6	> 1 000	480	> 1 000	137
4	[Aib^{24}, Ala^{31}, Aib^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSA**B**RHYINL**AB**RQRY	4166.9	4167.6	> 1 000	n. d.	>1 000	14.4
5	[Aib^{24}, Aib^{28}, Ala^{31}, Aib^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSA**B**RHY**B**NL**AB**RQRY	4139.1	4139.6	> 1 000	n. d.	>1 000	70.5
6	[hPP$^{1-7}$, Ala^{31}, Aib^{32}]-pNPY	APLEPVYPGEDAPAEDLARYYSALRHYINL**AB**RQRY	4163.2	4163.7	> 1 000	n. d.	> 1 000	6.1
7	[hPP$^{1-17}$, Ala^{31}, Aib^{32}]-pNPY	APLEPVYPGDNATPEQMARYYSALRHYINL**AB**RQRY	4207.7	4209.8	> 1 000	n. d.	192	0.9
8	[pNPY$^{1-7}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP	YPSKPDNPGDNATPEQMAQYAADLRRYINM**AB**RQRY	4185.8	4186.7	> 1 000	n. d.	920	12.1
9	[pNPY$^{1-7, 19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP	YPSKPDNPGDNATPEQMARYYSALRRYINM**AB**RQRY	4278.3	4278.8	500	n. d.	81	0.8
10	[cPP$^{1-7}$, pNPY$^{19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP	GPSQPTYPGDNATPEQMARYYSALRRYINM**AB**RQRY	4208.3	4207.8	620	n. d.	50	0.2
11	[cPP$^{1-7}$, pNPY$^{19-23}$, Ala^{31}, Aib^{32}, His^{34}]-hPP	GPSQPTYPGDNATPEQMARYYSALRRYINM**AB**RHRY	4216.1	4216.8	560	n. d.	27	3.6
12	[Ala^{31}, Pro^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**AP**RQRY	4206.8	4207.7	> 1 000	666	> 1 000	118
13	[Ala^{31}, D-Pro^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**Ap**RQRY	4207.4	4207.7	> 1 000	>1 000	2 000	>1000
14	[Ala^{31}, Hyp^{32}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**AU**RQRY	4223.3	4222.7	> 1 000	1 000	4 000	157
15	[Ala^{31}, Aib^{34}]-pNPY	YPSKPDNPGEDAPAEDLARYYSALRHYINL**A**TR**B**RY	4167.9	4168.6	1 000	1 000	99	20
16	[Ala^{31}, Aib^{32}]-pNPY (2-36)	PSKPDNPGEDAPAEDLARYYSALRHYINL**AB**RQRY	4031.7	4032.5	> 1 000	n.d.	> 1 000	7
17	[Ala^{31}, Aib^{32}]-pNPY (18-36)	ARYYSALRHYINL**AB**RQRY	2398.1	2398.8	325	39	307	>1000
18	[Ahx$^{5-24}$, Ala^{31}, Aib^{32}]-pNPY	YPSK**X**RHYINL**AB**RQRY	2162.4	2162.6	951	65	901	>1000

[a] Ala^{31} is shown in boldface; Aib is indicated as B, D-Pro as p, Hyp as U, and Ahx as X. The hPP positions introduced into NPY are underlined with a solid line; the pNPY positions introduced into hPP are underlined with a dotted line, and the cPP positions introduced into hPP are underlined with a broken line.

2−3 times in triplicate. SD was ±20% of the mean value, if not otherwise indicated. IC_{50} values of the binding curves were calculated by nonlinear regression on a one-site competition based model using Prism 3.0 (Table 1).

RESULTS

Peptide Synthesis. The amino acid sequence of the NPY analogues and of the PP/NPY chimera are shown in Table 1. The synthesis was accomplished by multiple solid-phase technique using the Fmoc/*tert*-butyl chemistry and HOBt/DIC as coupling reagents. The acylation of Aib by Ala, Leu, or Tyr went smoothly and was run to completion without the need of special and strong activating reagents. Only the sequence Ala-Aib-Ala-Aib at positions 29−32 of peptide **3** turned out to be more difficult, as shown by the presence of a minor peak (15%) in the analytical HPLC chromatogram of the crude product (data not shown). The impurity was found to have a mass difference of −156 that was attributed to Ala-Aib.

Methionine was introduced into the peptide chain without side-chain protection as usual in Fmoc-strategy. The analogues were characterized by analytical HPLC and ESI-MS. The HPLC chromatograms and the ESI-MS spectra of the crude methionine-containing peptides (**7**−**11**) revealed the presence of side products that corresponded to the methionine sulfoxide which was formed during the peptide chain assembly by multiple peptide synthesis or the TFA cleavage. The methionine sulfoxide was completely reduced by using trimethylbromosilane in the presence of ethanedithiol under acidic conditions (TFA) (*17*).

Receptor Binding Affinity. Binding of the peptides to the Y-receptors was tested by using a competition assay on cell lines selectively expressing the Y_1-, Y_2-, Y_4-, or Y_5-receptor subtypes (*9*). The binding affinity is reported as the IC_{50} value in Table 1. The full-length NPY analogues that contain the motif Ala^{31}−Aib^{32} (**1**−**5**) were found to be selective at the Y_5-receptor, with an affinity that was in the range of 5.9−137 nM. The IC_{50} values at the Y_2-receptor were >500 nM, and the binding potency at the receptors Y_1 and Y_4 was in the micromolar range. The only exception was peptide **2**, [Ala^{31}, Aib^{32}, Pro^{34}]-pNPY, which had a reduced Y_5-receptor selectivity relative to the Y_4-receptor. Accordingly, it bound to the Y_4- and Y_5-receptors with an affinity of 260 nM and 16.4 nM, respectively.

The presence of one or two Aib residues in the α-helical region 24−28 led to reduced affinity (14.4 nM for peptide **4** and 70.5 nM for peptide **5**). For the NPY analogue **3**, in which the motif Ala-Aib was repeated twice consecutively at positions 29−32, only moderate affinity was found at the Y_5-receptor (137 nM).

The NPY chimera **6** and **7** contain the N-terminal part of hPP, the segments 1−7 and 1−17, respectively. While the binding profile of peptide **6** was very similar to that of the analogue **1**, peptide **7** turned out to be 5- to 6-fold more potent at the receptors Y_4 and Y_5, thus displaying very high Y_5-receptor affinity (IC_{50} 0.92 nM) as well as selectivity.

Among the PP chimera **8**−**11**, peptides **9** and **10** turned out to bind to the Y_5-receptor with a subnanomolar affinity (0.82 nM and 0.20 nM, respectively), while only with moderate affinity to the Y_4- (>50 nM) and Y_1- (>500 nM) receptor. The modification of peptide **10** by the replacement of Gln^{34} with His (analogue **11**) led to a partial loss of affinity at the Y_5-receptor (18-fold) but to a slight increase at the Y_4-receptor (2-fold), with a subsequent loss of specificity for the Y_5-receptor. The analogue [pNPY$^{1-7}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (**8**) was less potent at the Y-receptors as compared to the other PP chimera. By comparison of the amino acid sequences of peptides **8** and **9**, the only difference is the

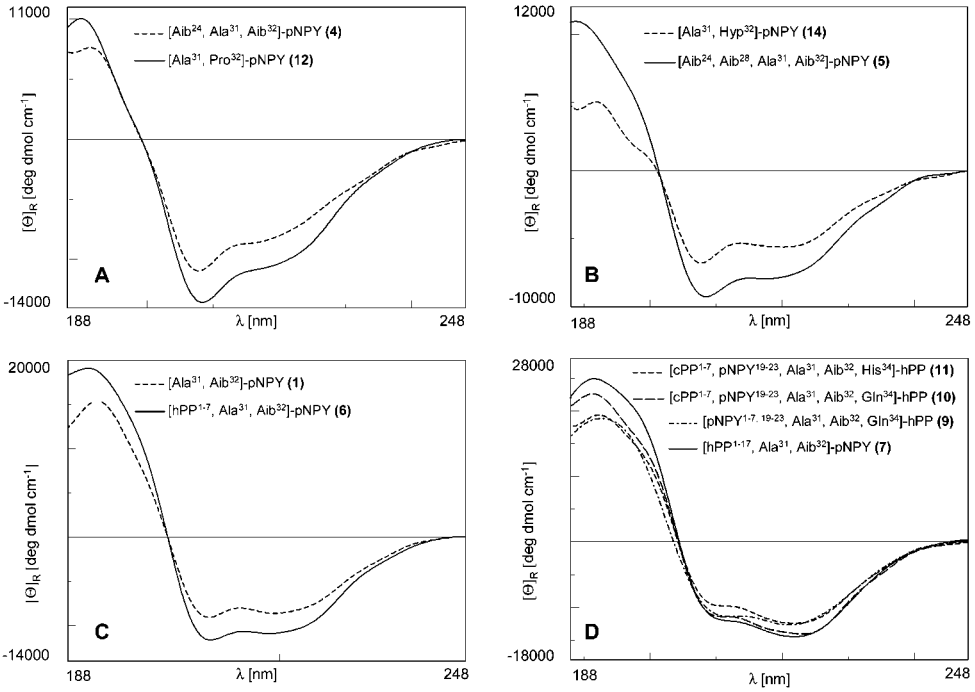

FIGURE 1: CD spectra of the NPY analogues and PP/NPY chimera in aqueous solution, at pH 7 and 20 °C. (Panel A) The ratio between the ellipticity at 220 and 208 nm (*R*) is 0.74−0.76, and the Y_5-receptor affinity is 14−118 nM. (Panel B) *R* is 0.82−0.85, and the Y_5-receptor affinity is 70−157 nM. (Panel C) *R* is 0.94−0.96, and the Y_5-receptor affinity is 4−6 nM. (Panel D) *R* is 1.15−1.32, and the Y_5-receptor affinity is 0.2−3.56 nM.

absence of the pNPY segment 19−23 in **8**. The lack of this primary structure element in the analogue **8** led to a reduction of affinity of 15-fold at the Y_5-receptor, 11-fold at the Y_4-receptor, and 4-fold at the Y_1-receptor.

The two NPY analogues containing Ala at position 31 and Pro or Hyp at position 32 (peptides **12** and **14**) turned out to be Y_5-receptor-selective as the corresponding NPY analogues containing Aib at position 32; however, significant loss of affinity was found for both peptides. The introduction of D-Pro caused a dramatic loss of affinity at the Y_5-receptor (>1000 nM for [Ala^{31}, D-Pro^{32}]-pNPY versus 118 nM for [Ala^{31}, Pro^{32}]-pNPY).

The shift of the Aib-substitution from position 32 to position 34 (peptide **15**) reduced the Y_5-receptor affinity but enhanced the Y_4-receptor one; this led to a lack of Y_5-receptor specificity relative to the Y_4-receptor but not to the Y_1- and Y_2-receptor subtypes.

Circular Dichroism. The conformation of peptides **1**−**16** was investigated in aqueous solution at neutral pH by using circular dichroism (CD) spectroscopy. As shown in Figure 1, all peptides adopted a helical structure; however, the CD profiles were different in the relative intensity of the two negative bands at 220 and 208 nm. The analogues [Aib^{24}, Ala^{31}, Aib^{32}]-pNPY (**4**) and [Ala^{31}, Pro^{32}]-pNPY (**12**) showed a minimum at 208 nm and a shoulder at 220 nm, and the ratio of the ellipticity at 220 nm versus the ellipticity at 208 nm (*R*) was 0.74 for the first peptide and 0.76 for the second one (Figure 1, panel A). Surprisingly, the CD spectrum of [Ala^{31}, D-Pro^{32}]-pNPY (**13**) turned out to be superimposable to that of the corresponding analogue containing the L-isomer of Pro (data not shown). We attribute this to the helix-breaking property of Pro as well as D-Pro and believe that both molecules are disordered at their C-termini in solution. The CD curves of the peptides [Aib^{24}, Aib^{28}, Ala^{31}, Aib^{32}]-pNPY (**5**) and [Ala^{31}, Hyp^{32}]-pNPY (**14**) were characterized by an *R* value of 0.85 and 0.82, respectively (Figure 1, panel B). The CD spectra of [Ala^{31}, Aib^{32}]-pNPY (**1**) and of the NPY chimera containing the hPP segment 1−7, [hPP$^{1-7}$, Ala^{31}, Aib^{32}]-pNPY (**6**) had a higher *R* value, which was 0.96 for peptide **1** and 0.94 for peptide **6** (Figure 1, panel C). The shortened NPY analogue [Ala^{31}, Aib^{32}]-pNPY (2−36) (**16**) gave a CD profile similar to that of the corresponding full-length analogue, with the same *R* value. $R > 1$ was found for the hPP analogues **9**−**11** and for the NPY chimera [hPP$^{1-17}$, Ala^{31}, Aib^{32}]-pNPY (**7**) ($1.15 \leq R \leq 1.32$) (Figure 1, panel D).

The two NPY analogues containing Pro or Aib at position 34, [Ala^{31}, Aib^{32}, Pro^{34}]-pNPY (**2**), and [Ala^{31}, Aib^{34}]-pNPY (**15**) and the PP chimera **8** were characterized by CD spectra displaying some differences as compared to the CD profiles described previously. In Figure 2, panel A, the CD curves of peptides **2** and **15** are reported and compared to that of the analogue [Ala^{31}, Aib^{32}]-pNPY (**1**); the additional presence of Pro^{34} in **2** caused a blue-shift of the negative band at 220 nm as well as an increase in its intensity, and the *R* value rose from 0.96 to 1.02. In contrast, the movement of Aib from position 32 to position 34 resulted in a red-shift of the CD spectrum and also increased the *R* value from 0.96 to 1.06. In Figure 2, panel B, the CD spectrum of the hPP analogue [pNPY$^{1-7}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (**8**) is compared to that of the corresponding analogue, where the hPP fragment 19−23 was replaced by that of pNPY (peptide **9**). The most striking difference between the two curves was the more pronounced negative band at 208 nm for the peptide lacking the central pNPY segment (**8**); accordingly, the *R* value decreased from 1.15 to 1.06.

DISCUSSION

From structure−affinity and structure−activity relationship studies it is known that the C-terminus of NPY and of the

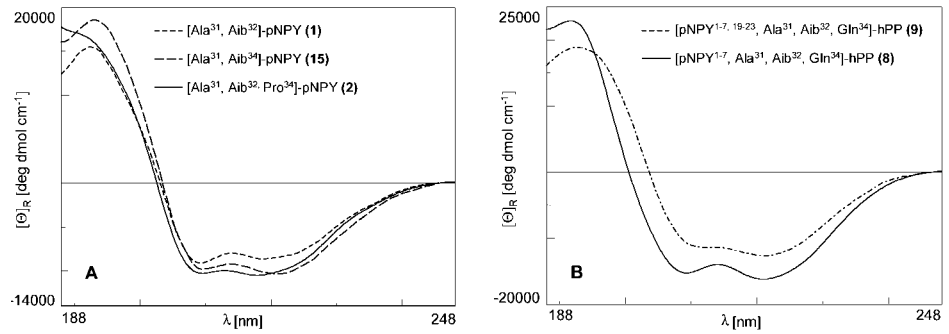

FIGURE 2: CD spectra of the NPY analogues [Ala^{31}, Aib^{32}, Pro^{34}]-pNPY (**2**) and [Ala^{31}, Aib^{34}]-pNPY (**15**), and comparison with [Ala^{31}, Aib^{32}]-pNPY (**1**) (panel A). CD spectrum of [$pNPY^{1-7}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (**8**) and comparison with [$pNPY^{1-7,19-23}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (**9**) (panel B).

other members of the NPY family represents the functional part of the ligands. Amino acid replacements as well as chemical modifications that involve the C-terminal pentapeptide are poorly tolerated at all NPY receptors. In particular, the two arginine side-chains at positions 33 and 35 play a key role in the binding and activation of the four receptor subtypes Y_1, Y_2, Y_4, and Y_5 (*19*). In addition, the C-terminal amide is essential, as suggested by the complete loss of affinity after its conversion to a carboxy group or ester (*20*). The analogue [Ala^{34}]-pNPY is as potent as NPY at the Y_4- and Y_5-receptor subtypes, while some affinity is lost at the Y_1- and Y_2-receptors (*19*). Pro^{34} has been shown to be perfectly tolerated at the receptors Y_1, Y_4, and Y_5 but not at the Y_2-receptor (*2*, *21*). The receptors Y_1, Y_2, and Y_4 were sensitive to the Ala-substitution at position 32 ($Y_1 > Y_2 > Y_4$), with a loss of affinity in the range of 70−3000-fold, while the affinity at the Y_5-receptor was reduced only 10-fold (*19*).

So far, there is no information about the bioactive conformation adopted by the C-terminus of NPY in the binding pocket of the different NPY receptors. The solution structure of NPY has been investigated by circular dichroism, 2D-NMR combined with molecular dynamics, and molecular modeling based on the crystal structure of avian PP (aPP). It is well-established that there is an amphipatic α helix in the C-terminal region (19−34 (*22*), 15−35 (*23*), 11−36 (*24*), 13−36 (*25*)). In the crystalline form of aPP (*26*) and in some NMR studies (*22*), the C-terminal end is structurally not well-defined. The observation that proline is tolerated at position 34 by all receptors with the exception of the Y_2-receptor suggests that a turn motif might be present in the bioactive conformation of the ligand at the receptors Y_1, Y_4, and Y_5. Therefore, we decided to introduce a turn-inducing motif into positions 31 and 32, which are the residues next to the very sensitive C-terminal tetrapeptide. We chose the dipeptide Ala-Aib because of its property to induce *β*-turns, especially 3_{10}-helical turns (*27*, *28*). [Ala^{31}, Aib^{32}]-pNPY has been recently investigated by 2D-NMR (*7*): the most striking difference between this analogue and native NPY has been identified in the C-terminal nonapeptide that was α-helical in NPY, while in the analogue a 3_{10}-turn in the region 28−31 was present, followed by a flexible carboxy end. Interestingly, the presence of the key motif Ala^{31}−Aib^{32} conferred high selectivity of NPY analogues for the Y_5-receptor. The presence of further Aib residues in the region 24−30 reduced the affinity at all Y-receptors. The combination of Ala^{31}−Aib^{32} with Pro^{34} modulated the Y_4- and Y_5-receptor affinity; while the Y_4-receptor affinity was improved, the Y_5-receptor affinity was slightly decreased. The loss of specificity with respect to the Y_4-receptor may be attributed to Pro^{34}, which is present in most species of PP (*29*). The results obtained with the (Ala-Aib)-containing NPY analogues lead to the conclusion that a C-terminal turn-inducing element provides selectivity for the Y_5-receptor. Thus, the next step is the improvement of affinity at this subtype without loosing specificity.

In a recent work, we developed a series of PP/NPY chimera, some of which turned out to be highly potent at the Y_5-receptor (up to 15-fold more potent than NPY itself); however, they had no selectivity (*30*). Therefore, we decided to combine the primary sequence of the highly potent chimera with the key motif Ala-Aib, to obtain peptides with high affinity as well as high selectivity at the Y_5-receptor, which would prove the concept of the turn-inducing motif as the key structure for selectivity. As expected, most of the (Ala-Aib)-containing chimera bound to the Y_5-receptor with high affinity, which was comparable to that of NPY for the analogues **7** and **9** and even 3-fold higher for the analogue **10**. His^{34} in the analogue **11** turned out to be less favorable at the Y_5-receptor than Gln^{34} (analogue **10**), as peptide **11** had 18-fold lower affinity than peptide **10**. Instead, the affinity at the Y_4-receptor increased almost 2-fold in the presence of His^{34}, with consequent loss of specificity of the peptide **11** for the Y_5-receptor. Furthermore, the hPP chimera lacking the central pNPY segment 19−23 (peptide **8**) was characterized by a reduced affinity at the receptors Y_4 and Y_5 as compared to the corresponding hPP analogue that contained pNPY 19−23. This suggests that the sequence 19−23 of pNPY also plays an important role in inducing the bioactive conformation. By comparing the two NPY chimera that contain the hPP sequences 1−7 or 1−17, it was observed that the longer hPP segment favored the binding at the Y_4- and Y_5-receptor subtypes; however, the peptide [hPP^{1-17}, Ala^{31}, Aib^{32}]-pNPY remained selective for the Y_5-receptor.

To prove the hypothesis that a turn-inducing motif may be used for selectivity at the Y_5-receptor, we investigated the effect of Pro at position 32. Interestingly, also the analogue [Ala^{31}, Pro^{32}]-pNPY (**12**) showed a Y_5-receptor preferring binding profile. When L-Pro^{32} was substituted with the D-isomer (peptide **13**), the Y_5-receptor affinity decreased strongly. This suggests that the stereochemistry of the turn-inducing residue 32 is of major importance for the binding

conformation. Furthermore, we tested the influence of the *trans*-4-hydroxy group of hydroxyproline on receptor binding (peptide **14**). The affinity at the Y_5-receptor was comparable to that of the analogue [Ala^{31}, Pro^{32}]-pNPY. The similarity of the binding profiles of [Ala^{31}, Pro^{32}]-pNPY (**12**) and [Ala^{31}, Aib^{32}]-pNPY is reflected in structural data obtained by 2D-NMR spectroscopy (see ref *7* for [Ala^{31}, Aib^{32}]-pNPY and the accompanying paper for peptide **12**). According to the membrane compartment theory originally developed by Schwyzer (*31*) and Moroder et al. (*32*), we believe that membrane association of the ligand hormone is an essential step preceding receptor binding. In contrast to native pNPY with its α helix extending up to the C-terminus the C-terminal tetrapeptide of the two Y_5-receptor-selective mutants is much more flexible, both when bound to DPC micelles (*33*) and in solution (*25*).

To investigate the importance of the position of the Aib residue in the C-terminal sequence, we shifted Aib from position 32 to the positions 24, 30, and 34. We found a loss of Y_5-receptor affinity as well as selectivity relative to the Y_4-receptor. This suggests that position 32 may be highly specific for the Y_5-receptor.

By the analysis of the solution structure of the Aib-containing analogues by circular dichroism, it was possible to correlate the conformational and biological properties of the peptides and to classify them in four different groups (Figure 1, panels A−D). (A) Peptides **4** and **12** showed a Y_4-receptor affinity of >1000 nM and a Y_5-receptor affinity of 14−118 nM; they were characterized by a similar helical CD profile, with a ratio (*R*) of the ellipticity at 220 nm versus the one at 208 nm of 0.74−0.76. (B) The analogues **5** and **14** also bound to the Y_4-receptor in the micromolar range, but their Y_5-receptor affinity was reduced to 70.5−157 nM. Their CD spectra presented a very similar *R* value that was 0.82−0.85. (C) A micromolar affinity at the Y_4-receptor and an affinity in the range of 6 nM at the Y_5-receptor were found for the peptides **1** and **6**. The *R* value obtained from their CD curves was in the range of 0.94−0.96. (D) Finally, the hPP analogues **9**−**11** and the NPY chimera **7** were the most potent ligands at the Y_5-receptor (0.2−3.6 nM), and bound to the Y_4-receptor with affinities which were in the range of 27.3−167 nM. The *R* value from the CD spectra of this group was determined to be >1.15.

The analogues [Ala^{31}, Aib^{32}, Pro^{34}]-pNPY (**2**), [Ala^{31}, Aib^{34}]-pNPY (**15**), and [pNPY$^{1-7}$, Ala^{31}, Aib^{32}, Gln^{34}]-hPP (**8**) could be classified in none of the four groups previously described: the peptide **2** was similar to the members of the group C with respect to its affinity at the Y_5-receptor; however, it turned out to be at least 4-fold more potent at the Y_4-receptor. The peptide **15** showed a Y_5-receptor affinity comparable to that of the members of the group A, but it was at least 10-fold more potent at the Y_4-receptor. Both the peptides **2** and **15** showed an increase in the intensity of the band at 220 nm ($R = 1.02-1.06$) (Figure 2, panel A). The hPP chimera **8** showed a much lower affinity at the Y_5-receptor than the other chimera (at least 3-fold and up to 60-fold lower) and also a more reduced affinity for the Y_4-receptor. To the different biological behavior corresponded a different CD profile, characterized by a more pronounced band at 208 nm ($R = 1.06$), as shown in Figure 2, panel B.

CONCLUSIONS

We developed a new class of Y_5-receptor specific ligands which are characterized by the presence of a turn-inducing element at position 32. We found that Aib as well as Pro in this position induce selectivity at the Y_5-receptor, with Aib being more suitable than Pro. The NMR structure of [Ala^{31}, Aib^{32}]-pNPY showed a 3_{10}-turn involving residues 28−31. This may suggest that the specific conformation adopted by the ligand in this region is of major importance for receptor recognition and binding. On the basis of this supposition, we are currently carrying out studies based on peptide and peptidomimetics design, to further characterize the structural requirements for high affinity and specificity at the Y_5-receptor. These selective analogues are very interesting also for their ability to act as agonists of NPY at the Y_5-receptor; they have been recently shown to stimulate food intake in rats (*7*). Accordingly, they are promising tools to understand the role of NPY as orexigenic neuropeptide.

REFERENCES

1. Tatemoto, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5485−5489.
2. Michel, M. C., Beck-Sickinger, A., Cox, H., Doods, H. N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., and Westfall, T. (1998) *Pharmacol. Rev. 50*, 143−150.
3. Gregor, P., Feng, Y., DeCarr, L. B., Cornfield, L. J., and McCaleb, M. L. (1996) *J. Biol. Chem. 271*, 27776−27781.
4. Inui, A. (1999) *Trends Pharmacol. Sci. 20*, 43−46.
5. Gerald, C., Walker, M. W., Criscione, L., Gustafson, E. L., Batzl-Hartmann, C., Smith, K. E., Vaysse, P., Durkin, M. M., Laz, T. M., Linemeyer, D. L., Schaffhauser, A. O., Whitebread, S., Hofbauer, K. G., Taber, R. I., Branchek, T. A., and Weinshank, R. L. (1996) *Nature 382*, 168−171.
6. Wyss, P., Stricker-Krongrad, A., Brunner, L., Miller, J., Crossthwaite, A., Whitebread, S., and Criscione, L. (1998) *Regul. Pept. 75*, 363−371.
7. 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.
8. Rudolf, K., Eberlein, W., Engel, W., Wieland, H. A., Willim, K. D., Entzeroth, M., Wienen, W., Beck-Sickinger, A. G., and Doods, H. N. (1994) *Eur. J. Pharmacol. 271*, R11−R13.
9. Wieland, H. A., Engel, W., Eberlein, W., Rudolf, K., and Doods, H. N. (1998) *Br. J. Pharmacol. 125*, 549−555.
10. Morgan, D. G., Small, C. J., Abusnana, S., Turton, M., Gunn, I., Heath, M., Rossi, M., Goldstone, A. P., O'Shea, D., Meeran, K., Ghatei, M., Smith, D. M., and Bloom, S. (1998) *Regul. Pept. 75*, 377−382.
11. Haynes, A. C., Arch, J. R., Wilson, S., McClue, S., and Buckingham, R. E. (1998) *Regul. Pept. 75*, 355−361.
12. Daniels, A. J., Matthews, J. E., Slepetis, R. J., Jansen, M., Viveros, O. H., Tadepalli, A., Harrington, W., Heyer, D., Landavazo, A., Leban, J. J., et al. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9067−9071.
13. Kanatani, A., Ishihara, A., Asahi, S., Tanaka, T., Ozaki, S., and Ihara, M. (1996) *Endocrinology 137*, 3177−3182.
14. Ishihara, A., Tanaka, T., Kanatani, A., Fukami, T., Ihara, M., and Fukuroda, T. (1998) *Am. J. Physiol. 274*, R1500−R1504.
15. Kanatani, A., Ito, J., Ishihara, A., Iwaasa, H., Fukuroda, T., Fukami, T., MacNeil, D. J., Van der Ploeg, L. H., and Ihara, M. (1998) *Regul. Pept. 75*, 409−415.
16. Criscione, L., Rigollier, P., Batzl-Hartmann, C., Rüeger, H., Stricker-Krongrad, A., Wyss, P., Brunner, L., Whitebread, S., Yamaguchi, Y., Gerald, C., Heurich, R. O., Walker, M. W., Chiesi, M., Schilling, W., Hofbauer, K. G., and Levens, N. (1998) *J. Clin. Invest. 102*, 2136−2145.
17. Beck, W., and Jung, G. (1994) *Lett. Pept. Sci. 1*, 31−37.
18. Ingenhoven, N., and Beck-Sickinger, A. G. (1997) *J. Recept. Signal Transduction Res. 17*, 407−418.
19. Eckard, C. P., Cabrele, C., Wieland, H. A., and Beck-Sickinger, A. G. (2001) *Molecules 6*, 448−467.

20. Hoffmann, S., Rist, B., Videnov, G., Jung, G., and Beck-Sickinger, A. G. (1996) *Regul. Pept. 65*, 61−70.
21. Fuhlendorff, J., Gether, U., Aakerlund, L., Langeland-Johansen, N., Thogersen, H., Melberg, S. G., Olsen, U. B., Thastrup, O., and Schwartz, T. W. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 182−186.
22. Mierke, D. F., Durr, H., Kessler, H., and Jung, G. (1992) *Eur. J. Biochem. 206*, 39−48.
23. Darbon, H., Bernassau, J. M., Deleuze, C., Chenu, J., Roussel, A., and Cambillau, C. (1992) *Eur. J. Biochem. 209*, 765−771.
24. Cowley, D. J., Hoflack, J. M., Pelton, J. T., and Saudek, V. (1992) *Eur. J. Biochem. 205*, 1099−1106.
25. Monks, S. A., Karagianis, G., Howlett, G. J., and Norton, R. S. (1996) *J. Biomol. NMR 8*, 379−390.
26. Blundell, T. L., Pitts, J. E., Tickle, I. J., Wood, S. P., and Wu, C.-W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4175−4179.
27. Möhle, K., Gussmann, M., and Hofmann, H. J. (1997) *J. Comput. Chem. 18*, 1415−1430.
28. Toniolo, C., Polese, A., Formaggio, F., Crisma, M., and Kamphuis, J. (1996) *J. Am. Chem. Soc. 118*, 2744−2745.
29. Larhammar, D. (1996) *Regul. Pept. 62*, 1−11.
30. Cabrele, C., Wieland, H. A., Langer, M., Stidsen, C., and Beck-Sickinger, A. G. (2001) *Peptides 22*, 365−378.
31. Schwyzer, R. (1991) *Biopolymers 31*, 785−792.
32. Moroder, L., Romano, R., Guba, W., Mierke, D. F., Kessler, H., Delporte, C., Winand, J., and Christophe, J. (1993) *Biochemistry 32*, 13551−13559.
33. Bader, R., Bettio, A., Beck-Sickinger, A. G., and Zerbe, O. (2001) *J. Mol. Biol. 305*, 307−329.

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