Y_1 and Y_2 Receptor Selective Neuropeptide Y Analogues: Evidence for a Y_1 Receptor Subclass†

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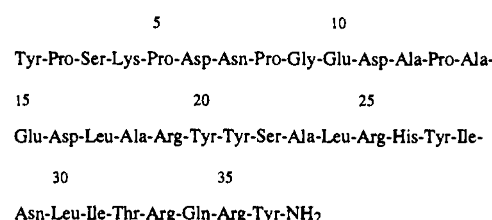
Neuropeptide Y (NPY), a 36-residue polypeptide produced abundantly in both nervous and peripheral tissues, appears to play a significant role in the regulation of diverse biological processes, including feeding behavior and cardiovascular and psychotropic functions. The actions of NPY are mediated through effective binding to specific receptors of which two, designated Y_1 and Y_2, have been well characterized. A shortened cyclic analogue of NPY, des-AA^{10-17}-*cyclo*-7/21[$Cys^{7,21}$]NPY,[1] was shown to retain high affinity for both human neuroblastoma SK-N-MC and SK-N-BE2 cell types (expressing Y_1 and Y_2 receptors, respectively). Increasing the size of the ring (des-AA^{10-17}-*cyclo*-2/27[$Cys^{2,27}$]NPY) in the present study produced a high-affinity analogue (K_i = 3.0 vs 0.3 nM for NPY) that bound exclusively to Y_2 receptors. Using the feedback from structure–activity relationships, we also describe the optimization of specific substitutions and bridging arrangements leading to the production of other truncated, high-affinity Y_1 selective analogues which bind, as does NPY itself, in the low-nanomolar range. Of greatest significance, des-AA^{10-17}-*cyclo*-7/21[$Cys^{7,21}$,Pro^{34}]NPY (**11**) was found to possess agonistic properties with an affinity comparable to that of the native NPY molecule when tested for its ability to inhibit norepinephrine-stimulated cAMP release in SK-N-MC human neuroblastoma cells. Compound **11** also caused an increase in blood pressure in anesthetized rats. However, in two central nervous system models of Y_1 receptor function, stimulation of feeding and anxiolytic activity, this analogue was inactive, which suggests the presence of a new subclass of receptors. In summary, the present results demonstrate that residues 10–17 of NPY are not directly involved in either Y_1 or Y_2 receptor recognition or activation. This suggests that the selectivity of NPY receptors is highly dependent on subtle conformational changes such as the substitution of residue 34 to a proline or the introduction of intramolecular constraints. Additionally, we have produced an analogue of NPY that selectively activates peripheral NPY Y_1 receptors.

Introduction

Neuropeptide Y (NPY, sequence of porcine NPY shown below), a member of the pancreatic polypeptide (PP) family, is a 36-residue C-terminally amidated polypeptide.[2,3] NPY has been demonstrated to elicit numerous pharmacological effects, including mediation of vasoconstriction, analgesia, anxiolysis, feeding behavior, and hormone secretion.[4–9] The sequence of NPY shows 69% homology to the intestinal hormone peptide YY (PYY) and 50% homology to PP. X-ray studies, spectral studies, and molecular dynamics simulation suggest that the tertiary structure of NPY may be characterized as a poly(proline) type II helix for residues 1–8, a β-turn through positions 9–14, an amphipathic α-helical segment for residues 15–32, and a nonstructured C-terminus (residues 33–36).[10–13]



The distribution of NPY is widespread throughout the periphery as well as in most brain regions. NPY is costored and coreleased with norepinephrine in sympathetic fibers. The best characterized forms of NPY receptors have been designated Y_1 and Y_2 receptors, though other forms have been proposed (for full review, see Gehlert, 1994).[14] In the sympathetic nervous system, receptors of the Y_1 subtype mediate vasoconstriction postsynaptically, while those of the Y_2 type act presynaptically in the regulation of catecholamine release.[7] The Y_1 receptor, the only type yet cloned,[15] mediates many of the actions that have been ascribed to NPY. The Y_2 subtype is the predominant form in the central nervous system. Both receptors are coupled to the inhibition of adenylate cyclase. The distinction between the two types is derived from differential

† The abbreviations for the amino acids are in accord with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9–37). The symbols represent the L-isomer except when indicated otherwise. Abbreviations: Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-l-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DCM, dichloromethane; DIEA, diisopropyethylamine; DME, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; Dpr, 2,3-diaminopropionic acid; DIC, 1,3-diisopropylcarbodiimide; EDT, ethanedithiol; HF, hydrofluoric acid; HOBt, 1-hydroxybenzotriazole; IBMX, 3-isobutyl-1-methylxanthine; NPY, neuropeptide Y; PP, pancreatic polypeptide; PYY, peptide YY; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA, triethylamine.

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binding properties of C-terminal fragments and modifications of NPY, especially the 13–36 fragment which binds only to Y_2 receptors and [Leu^{31},Pro^{34}]NPY which has been demonstrated to be a Y_1 selective agonist.[15–18] Additionally, several cyclic and centrally truncated analogues of NPY have been developed that show high selectivity for the Y_2 receptors.[1,19]

Because des-AA$^{10-17}$-*cyclo*-7/21[Cys^{7,21}]NPY (**6**) has high affinity for both receptor types, it was our goal to make modifications in the structure that would produce selectivity for the Y_1 receptor. Fuhlendorff *et al.* showed, in 1990, that [Leu^{31},Pro^{34}]NPY bound with high affinity to Y_1 receptor–containing cells while failing to be recognized by Y_2 receptors.[18] We hypothesized that by placing these substitutions in **6** and related truncated analogues we could produce potent compounds with preference for the Y_1 receptor without loss of affinity.

Results

Binding of Centrally Truncated Linear NPY Analogues. Analogues lacking central residues 10–17 without the addition of further stabilizing forces (**2**–**5**) all showed loss in binding effectiveness to both Y_1 and Y_2 receptors using the human neuroblastoma cell lines SK-N-MC and SK-N-BE2, respectively. Affinities for Y_1 receptors ranged from 77 to 120 nM compared to 2.0 nM for the full length NPY. Y_2 affinities ranged from 1.0 to 27 nM, compared to 0.3 nM for NPY. Neither N-terminal acetylation nor substitution in the central section was shown to profoundly effect binding when compared to the simple deletion analogue **2**. However, substitution of residues 7 and 21 by alanine produced **5** with a 10-fold loss of affinity for the Y_2 receptor, having a lesser effect on the Y_1 receptor.

Binding of Cyclo (7/21) Deletion Analogues. Constraining residues 7 and 21 in the centrally truncated analogue using a Cys–Cys side chain bridge (**6**) increased Y_1 and Y_2 binding affinities by 15- and 2-fold, respectively, when compared to the linear deletion analogue **2**. However, affinity of **7**, using a similar configuration except a longer Glu^7 to Lys^{21} lactam type bridge, was not significantly different from that of the corresponding linear **2**.

Binding of Centrally Branched Cyclo (2/21) Deletion Analogues. When a D-Lys^9(ϵ-acetyl) residue was incorporated (**8**) in the truncated and cyclized peptide, a similar binding profile was produced when compared to cyclic **6**. Inverting the chirality of the Cys bridgehead at residue 7 reduced the Y_1 affinity by 4-fold (**9**) while reducing the Y_2 affinity by less than 2-fold. However, when the chirality of the lysine branch was also inverted (**10**), Y_1 affinity was reduced by more than 26-fold, yet binding to Y_2 receptors was virtually unaffected.

Binding of Cyclo (2/27) Deletion Analogues. Cyclization between residues 2 and 27 in **16** and **17**, otherwise similar in size and sequence to previous analogues, produced selectivity for the Y_2 receptor with high affinity (3.0 nM) regardless of the chirality of the Cys bridgehead at position 27.

Binding of Pro^{34}-Substituted Cyclo (2/21) Deletion Analogues. In **11**–**15**, binding to Y_2 receptors was abolished when Gln^{34} was replaced by proline. Highest Y_1 affinity was observed (5.0 nM) in **11** when a Cys–Cys bridge was introduced, with the Asp-Dpr (**12**) and Glu-Lys (**13**) bridge arrangements having produced a 7- and 30-fold reduction in binding affinity, respectively. When leucine substituted the naturally occurring Ile^{31}, a 10-fold loss of affinity resulted despite the presence of the optimized Cys^7–Cys^{21} constraint. Further truncation of residues 18–20 (**15**) likewise reduced binding by almost 8-fold when compared to the optimized **11**. Figure 1 shows the specific displacement of [^{125}I]PYY from SK-N-MC and SK-N-BE2 cells by NPY and **11**.

cAMP Response to Des-AA$^{10-17}$-*cyclo*-7/21[Cys^{7,21},Pro^{34}]NPY. Compound **11**, in a dose dependent manner, decreased the norepinephrine-stimulated accumulation of cAMP in SK-N-MC cells. Results are comparable to that of NPY itself, indicating agonism. See Figure 2.

Effect of Des-AA$^{10-17}$-*cyclo*-7/21[Cys^{7,21},Pro^{34}]NPY on Mean Arterial Blood Pressure. Compound **11** was administered intravenously to urethane-anesthetized male Sprage–Dawley rats using venous catheters. The dose dependent increase in mean arterial pressure (MAP) measured 5 min following peptide administration is shown in Figure 3. Data for effects on arterial pressure are expressed as ΔMAP $\pm$ SEM from four rats.

Feeding Response to Des-AA$^{10-17}$-*cyclo*-7/21-[Cys^{7,21},Pro^{34}]NPY. The total intake of sweetened chow mixture by rats following infusion of **11** (6.15 $\pm$ 0.5 g, $N = 15$) was not statistically different from that recorded in rats given vehicle (4.73 $\pm$ 0.46 g, $N = 17$) using one-way ANOVA analysis and Fishers least significant difference test. NPY (17.35 $\pm$ 2.09 g, $N = 14$) was significantly different than vehicle ($p < 0.0001$). See Figure 4.

Discussion

NPY was first described in 1982.[2] Since then, significant changes in NPY levels have been suspected in the pathology of many disease states, including hypertension and obesity. Specific NPY receptors located in many central and peripheral tissues have been classified as Y_1, Y_2, and Y_3 receptors[22] on the basis of differences in binding properties of truncated and otherwise modified NPY analogues. Because NPY is a relatively large polypeptide with high homology with APP which has a well-defined tertiary structure by X-ray crystallography, elucidation of the minimum bioactive core of NPY would greatly assist the design of small, highly potent agonists or antagonists, ultimately leading to nonpeptide clinical candidates.

While deletion of N-terminal residues led to the loss of binding to the Y_1 receptor with retention of affinity to the Y_2 receptor, deletion of the C-terminal residues was detrimental to affinity at both receptors.[17] However, centrally truncated analogues which bind specifically and with high affinity to Y_2 receptors have been successfully developed.[1,19,20] On the basis of structure–activity relationship (SAR) studies by our group and others, it was hypothesized that central truncation of the hairpin loop may be tolerated by Y_1 receptors as well, especially if covalent molecular constraints were introduced. Exploration of several truncation possibilities indicated that modifications created by the deletion of residues 10–17 preserved low binding affinity at Y_1 receptor–expressing cells (human neuroblastoma SK-N-MC), though analogues with even larger truncations such as des-AA$^{7-24}$ were recognized by Y_2 receptors only, with high affinity.[1,19–22]

Table 1. Physicochemical and Biological Properties of NPY Analogues

no.	compd	CZE purity[a]	CZE t_R (min)[a]	mass[b] calcd	mass[b] obsd	K_i (nM) SK-N-MC	K_i (nM) SK-N-BE2
1	YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2 (NPY)	96	18.2	4254.74	4255.0	2.0	0.3
2	YPSKPDNPG ARYYSALRHYINLITRQRY-NH_2 des-AA^{10-17}-NPY	>85	16.3	3411.84	3411.8	77	2.5
3	Ac-YPSKPDNPk(Ac) ARYYSALRHYINLITRQRY-NH_2 des-AA^{10-17},Ac-[DLys^9(ε-Ac)]NPY	>85	18.6	3566.88	3566.6	87	1.8
4	Ac-YPSKPDNPk(Ac-Ala)ARYYSALRHYINLITRQRY-NH_2 des-AA^{10-17},Ac-[DLys^9(ε-Ac-Ala)]NPY	>95	18.8	3637.91	3637.6	96	1.0
5	YPSKPDAPG ARYASALRHYINLITRQRY-NH_2 des-AA^{10-17}[Ala^{7,21}]NPY	>95	16.2	3276.75	3277.1	120	27
6	YPSKPDCPG ARYCSALRHYINLITRQRY-NH_2 des-AA^{10-17}[Cys^{7,21}]NPY	>95	23.7	3339.66	3340.4	5.1	1.3
7	YPSKPDEPG ARYKSALRHYINLITRQRY-NH_2 des-AA^{10-17}[Glu^7,Lys^{21}]NPY	>95	16.1	3373.80	3373.9	100	9.0
8	YPSKPDCPGk(Ac) ARYCSALRHYINLITRQRY-NH_2 des-AA^{11-17}[DLys^{10}(ε-Ac),Cys^{7,21}]NPY	80	16.0	3440.00	3440.1	6.2	3.3
9	YPSKPDcPk(Ac) ARYCSALRHYINLITRQRY-NH_2 des-AA^{10-17}[DCys^7,DLys^9(ε-Ac),Cys^{21}]NPY	90	16.7	3451.76	3451.6	28	5.2
10	YPSKPDcPK(Ac) ARYCSALRHYINLITRQRY-NH_2 des-AA^{10-17}[DCys^7,Lys^9(ε-Ac),Cys^{21}]NPY	>90	16.2	3451.76	3452.0	160	5.4
11	YPSKPDCPG ARYCSALRHYINLITRPRY-NH_2 des-AA^{10-17}[Cys^{7,21},Pro^{34}]NPY	95	16.2	3307.67	3307.6	5.0	>1000
12	YPSKPDDPG ARYXSALRHYINLITRPRY-NH_2 des-AA^{10-17}[Asp^7,Dpr^{21},Pro^{34}]NPY	90	16.4	3286.73	3286.9	38	>1000
13	YPSKPDEPG ARYKSALRHYINLITRPRY-NH_2 des-AA^{10-17}[Glu^7,Lys^{21},Pro^{34}]NPY	>95	16.7	3342.80	3342.7	150	>1000
14	YPSKPDCPG ARYCSALRHYINLLTRPRY-NH_2 des-AA^{10-17}[Cys^{7,21},Leu^{31},Pro^{34}]NPY	>90	16.1	3340.86	3340.4	52	>1000
15	YPSKPDCPG YCSALRHYINLITRPRY-NH_2 des-AA^{10-20}[Cys^{7,21},Pro^{34}]NPY	>95	17.3	2917.47	2917.3	32	>1000
16	YCSKPDNPG ARYYSALRHCINLITRQRY-NH_2 des-AA^{10-17}[Cys^{2,27}]NPY	>92	15.7	3355.67	3355.8	>1000	3.0
17	YCSKPDNPG ARYYSALRHcINLITRQRY-NH_2 des-AA^{10-17}[Cys^2,DCys^{27}]NPY	>90	15.7	3355.67	3355.9	>1000	3.0

[a] Purity and retention time were determined by CZE using 0.1 M sodium phosphate, pH 2.5, and a voltage of 12 kV. Capillary was Supelco SElect P175 (50 cm × 75 μm). Detection was at 214 nm. Injections were for 4 s from stock solution at a concentration of 0.1 mg/mL. [b] Monoisotopic mass, m/z, measured with LSI-MS; glycerol and 3-nitrobenzyl alcohol (1:1) matrix; Cs ion source.

We presented preliminary results suggesting that with the adequate constraints, centrally truncated analogues of NPY could be designed that retained high affinity for the Y_1 receptor.[1] Noteworthy was the observation that bridging between residues 7 and 21 using a disulfide arrangement produced an analogue (**6**) equipotent with NPY, whereas the corresponding linear **2** showed greatly reduced affinities for both receptors.[23] Although the unconstrained **2** contained all the significant residues that compose the theoretical binding pharmacophore, disruption of the central hairpin loop removed the cohesive forces that bring together components of the Y_1-binding site. The Y_2 receptor, which has been shown to interact with short C-terminal fragments of NPY, recognized the linear analogue with relatively high affinity (2.5 nM) similar to that which

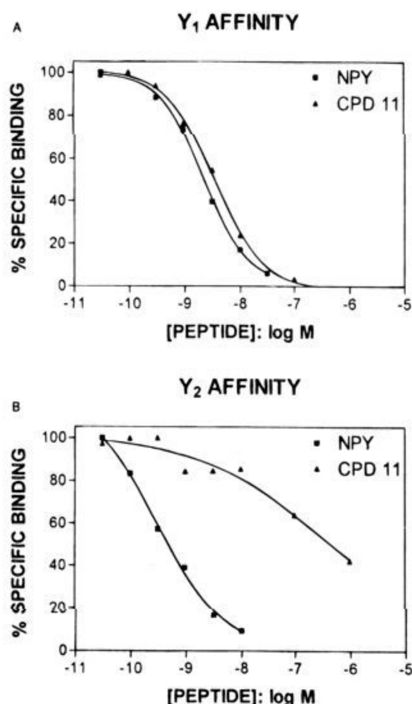

Figure 1. Displacement of [^{125}I]PYY from (A) SK-N-MC and (B) SK-N-BE2 cells by NPY and compound **11**. The error bars are smaller than the symbol sizes. The data are from a representative experiment performed in triplicate that was repeated twice with similar results.

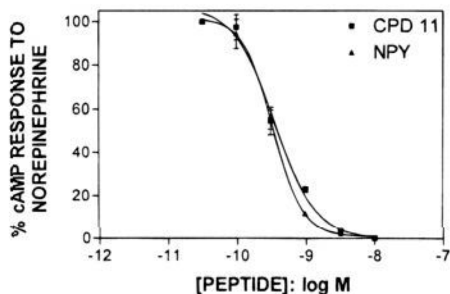

Figure 2. Effect of NPY and compound **11** to decrease norepinephrine-stimulated cAMP accumulation in SK-N-MC cells. The data are from a representative experiment that was replicated three times.

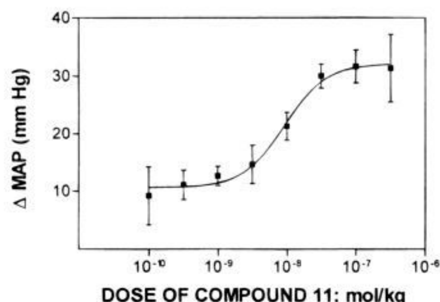

Figure 3. Effect of compound **11** on MAP in urethane-anesthetized rats. The injection artifact was 5−10 mmHg and was not subtracted from the data shown. The data are the mean and SEM from four rats.

would be expected by binding to the NPY$^{18-36}$ C-terminal fragment. The importance of functional groups presented by residues 7 and 21 (Gln and Tyr in the native sequence) was examined by the synthesis of an analogue containing Ala in those two positions (**5**).

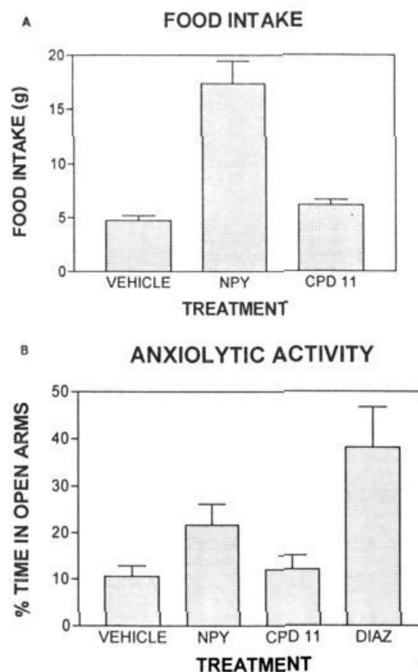

Figure 4. (A) Effect of NPY and compound **11** on food intake in rats. The peptides or vehicle was infused into the lateral ventricle, and food intake was measured 1.5 and 4 h postinjection. (B) Effects of NPY, compound **11**, and diazepam on time spent by rats in the open arms of an elevated plus maze. Observations were made 60 min after injection of NPY, compound **11**, or vehicle and 30 min after injection of diazepam. DIAZ: diazepam.

Binding affinities were reduced compared to **2**, especially at the Y_2 receptor. An earlier study showed that [Ala^7]NPY did not result in major loss of activity,[24] suggesting that the low affinity of **5** to SK-N-BE2 cells in the present study was likely the result of the loss of the phenolic side chain of position 21 which may be involved in the stabilization of the PP fold conformation.[25] It was speculated that introduction of the 7−21 bridge would bring the N- and C-termini into close proximity and thus adopt a conformation similar to that of the full length NPY. By changing from the disulfide motif to a lactam bridge, we were able to explore the influence that bridge length exerted on affinity. The Glu−Lys lactam modification (**7**) was suspected to be either too long or flexible and did not result in any improvement over linear **2**, despite the added constraint. Specific distance between the C- and N-termini is likely to be crucial to optimum binding affinity. This parameter will be further discussed later in this section.

Changing chirality of residues within a peptide sequence, introduction of side chain to side chain bridges, and other constructs have been identified that may result in more potent and selective analogues. We have identified two positions in NPY (9 and 6) where the introduction of a D-amino acid is not detrimental to binding affinity[26] and as a result have investigated the possibility of restoring structure integrity in analogues with deleted internal segments (residues 10−17) by the addition of these residues (referred to as a branch) on the side chain of a D-lysine at position 9.[24] Branch extension in linear analogues did not produce a deleterious effect on binding, nor did it improve affinity.[24,27] We revisited this concept after the introduction of a single bridge between residues 7 and 21 which resulted in **8**

with higher affinity to the Y_1 receptor while slightly decreasing the affinity to the Y_2 receptor when compared to that of linear **2** or **3**.

Because constrained **6** also bound with high affinity to the Y_1 receptor, we suspected that essential information needed for Y_1 receptor recognition was independent of the central α-helical segment or the specific residues contained therein. Rather, the spatial alignment of the two termini was thought to contribute most to high-affinity binding to the Y_1 receptor. Indeed, it appeared that the role of the central segment was to act as a template to present the peptide-binding site, since the extension of a side branch from the central region of an optimized analogue did not affect binding. When an acetylated D-lysine was substituted at position 9 in constrained **8**, binding to the Y_1 receptor was virtually unaffected compared to that of the parent **6**.

Using computer-modeling techniques on constrained **6**, the flexibility in the backbone of the conformation was hypothesized to permit the chirality of the bridgehead to be manipulated, yet Y_1 binding affinities of branched analogues **8** and **9** differed by 5-fold, indicating a preference for the LCys−LCys construct. It was also hypothesized that the DCys^7−Cys^{21} bridge produced a misalignment of the termini which disrupted the critical orientation of the chemical features that interacted with the Y_1 receptor.[1] When this unfavorable bridgehead orientation was combined with a sterically hindered branch orientation (**10**), binding to Y_1 receptors was severely disrupted. The position of the central branch extended from an L−lysine possibly prevented the termini of the molecule from approaching optimum spatial alignment. By contrast, **8**−**10** bound to Y_2 receptors in the low-nanomolar range (3.3−5.4 nM), without an apparent preference for any particular branch or bridgehead orientation. Such lack of discrimination between the structures of these analogues by the Y_2 receptor indicates a high degree of fit tolerance. Since the Y_2 receptor recognizes the C-terminal fragment NPY$^{18-36}$ at high affinity (1.8 nM), the N-terminal half of the molecule, together with the correct spatial alignment between the termini, provides the "fine tuning" or sub-nanomolar level of fit to the receptor.

Analogues of similar size (des-AA$^{10-17}$), constructed by bridging between residues 2 and 27, produced **16** and **17** with exclusive preference for the Y_2 receptor. Binding properties were unaffected by the chirality of the two cysteines composing the disulfide bridge. Modeling of both types of bridging arrangements indicated that both could adopt a folded conformation similar to that of the parent NPY. Placing a constraint closer to the terminal segments of the molecule locked the binding site into an imperfect fit for the Y_1 receptor. Alternatively, it is possible that the substitution of Tyr^{27} with the cysteine was in itself a deleterious modification. We know from previous studies that substitution by DTyr in position 27 resulted in an analogue devoid of affinity for the Y_1 receptor.[26] However, in a similar series of cyclic deletion analogues, we showed that a Glu^2 to DDpr^{27} bridge *increased* Y_1 affinity over similar unsubstituted analogues of the same size.[1] This indicated that substitution in these positions may be tolerated or overcome by effective constraint. In that study, other cyclo (2/27) analogues showed selectivity for the Y_2 receptor which was explained to be the result of specific bridge length or chirality of bridgeheads and the rigidity of the β-turn section. As the analogues produced in that series were of higher affinity and greater selectivity than the present analogues, we turned our attention to identifying compounds that would instead be selective for the Y_1 receptor with high affinity.

The best Y_1 selective NPY analogue developed so far was reported by Fuhlendorff *et al.*, in 1990, who demonstrated that the substitution of Leu^{31} and Pro^{34} in the full NPY sequence produced an analogue with exclusive preference for the Y_1 receptor, without loss of affinity.[18] It was hypothesized that if the conformation of a 10−17 truncated compound indeed closely mimicked the bioactive conformation of NPY, similar substitutions in the shortened sequence would produce Y_1 receptor selectivity. We found that des-AA$^{10-17}$-*cyclo*-7/21[Cys^{7,21},Leu^{31},Pro^{34}]NPY (**14**) did in fact bind exclusively to Y_1 receptors, though with a 10-fold loss in affinity (K_i = 52 nM) when compared to that of **6**. Since the substitution in position 31 was very conservative (*i.e.*, leucine for an isoleucine) and perhaps unnecessary for receptor specificity, des-AA$^{10-17}$-*cyclo*-7/21[Cys^{7,21}, Pro^{34}]NPY (**11**) was prepared, which demonstrated high-affinity binding to SK-N-MC cells (5.0 vs 2.0 nM for NPY; see Figure 1).

Hoping to further optimize the structural features of **11**, we focused primarily on changing the length, position, and chemical nature of the bridge. The use of a lactam linkage to bring the C- and N-termini together provided a synthetically convenient method for altering the length of the bridge. An Asp to Dpr bridge (Cα−Cα distance of 4.6Å as derived by computer modeling) represented a size equivalent lactam cyclic analogue as compared to the Cys−Cys-bridged compound (4.9 Å). Compound **12**, des-AA$^{10-17}$-*cyclo*-7/21[Asp^7,Dpr^{21},Pro^{34}]-NPY, bound exclusively to Y_1 receptors with a K_i of 38 nM. A much longer bridge (6.8 Å) was constructed by using a Glu to Lys lactam cycle. This analogue, **13**, had lower affinity (K_i = 150 nM) when compared to that of the parent disulfide or shorter lactam-containing analogues, indicating that a more constrained structure was optimal for Y_1 receptor recognition. The deletion of three additional residues (Ala^{18}, Arg^{19}, and Tyr^{20}), where a cystine bridge was constructed between positions 7 and 21, produced **15** which bound to Y_1 receptors with a K_i of 32 nM, indicating that residues 18−20 are not critical for high-affinity binding.

Having identified des-AA$^{10-17}$-*cyclo*-7/21[Cys^{7,21},Pro^{34}]-NPY (**11**) as the first truncated, high-affinity Y_1 specific peptide analogue developed yet, we sought to determine its biological profile. In a functional assay, **11** was shown to be equipotent with NPY in its ability to inhibit norepinephrine-stimulated cAMP accumulation in SK-N-MC human neuroblastoma whole cells and therefore determined to be a full potency agonist of NPY (see Figure 2). Likewise, a full *in vivo* agonist (hypertensive) response was produced when **11** was administered intravenously in the rat (Figure 3). Having confirmed that **11** indeed acted as a high-potency Y_1 receptor agonist both *in vitro* and in a peripheral model of Y_1-mediated activity, we sought further characterization of this compound in central nervous system-mediated functions.

Endogenous NPY in the hypothalamus has been shown to be a potent stimulator of feeding behavior even in satiated animals.[28,29] Likewise, [Leu[31],Pro[34]]NPY, a Y_1 receptor specific agonist, acts as a potent modulator of feeding behavior. Somewhat enigmatically however, des-AA$^{10-17}$-*cyclo*-7/21[Cys^{7,21},Pro^{34}]NPY failed to produce any effect on food consumption (Figure 4A). Also among the reported central actions of neuropeptide Y, it has been demonstrated that NPY may be an endogenous anxiolytic. Using the well-established plus maze conflict test,[30] we showed that preinfusion of NPY or diazepam in the lateral ventricles of rats increased time spent on open arms, supporting the hypothesis that NPY acts as an anxiolytic. Under the same set of experimental conditions, the full length Y_1 specific analogue [Leu^{31},Pro^{34}]NPY was shown to be virtually as effective as NPY in producing the anxiety-modifying action, and Y_2 receptor specific analogues were inactive, indicating a Y_1 receptor-mediated process (unpublished results). Again, des-AA$^{10-17}$-*cyclo*-7/21[Cys^{7,21},Pro^{34}]-NPY was shown to be inactive in this paradigm (Figure 4B).

Because both the anxiolytic and feeding effects of NPY are proposed to be mediated via postsynaptic Y_1 receptors in the brain, results derived from **11** possibly indicate the existence of a new subclass of the Y_1 receptors located in the lateral cerebral ventricle. This subclass appears to exhibit even more stringent structural requirements than the known "Y_1" receptor. Other investigators have speculated as to the possible existence of this as a yet uncharacterized receptor subpopulation.[14] A second possible explanation for the absence of activity by **11** after central administration could be that the path of degradation or elimination of this peptide in the brain, specifically an alteration of the cystine crossbridge, would likely yield a less effective Y_1 binder. Future investigations will include the evaluation of additional compounds from this present series to better elucidate the properties and function(s) of this new subclass of Y_1-like receptors.

In addition to the receptor selective NPY analogues produced in the present study, information gained so far has provided the basis for a more refined theory of the minimum bioactive conformation of NPY. The subtle chemical nuances that influence receptor specificity became apparent through the exploration of bridging options, specific substitutions, and chiral modifications, possibly revealing a new subclass of NPY receptor. Specifically, we have determined that des-AA$^{10-17}$-[Cys^{7,21},Pro^{34}]NPY selectively triggers only peripheral Y_1 receptors. Continuation of the present study will no doubt produce even smaller and more specific analogues that may find therapeutic application in the treatment of any number of the NPY-mediated disorders.

Materials and Methods

Peptide Synthesis. All peptides were synthesized manually using standard solid phase peptide synthesis (SPPS) techniques following Boc strategy on MBHA resins prepared in our laboratory by methods previously described.[1] Side chain protection of α-Boc amino acids was as follows: Arg(Tos), Asp(OcHx or OFm), Cys(*p*-MOB), Dpr(Fmoc), Glu(OcHx or OFm), His(Tos), Lys(2ClZ or Fmoc), Ser(Bzl), Thr(Bzl), and Tyr(2BrZ). The *t*-Boc protecting group was removed in a single 20 min treatment of 60% TFA/DCM in the presence of 1% anisole as a carbocation scavenger. Following neutralization using 10% TEA in DCM, Boc-protected amino acids were added to the growing peptide chain by a single coupling step using DIC as the coupling reagent. For difficult couplings and lactam cyclization on the resin, condensation steps were mediated via *in situ* active ester formation using BOP or HBTU in the presence of DIEA. The protected peptide-resin was cleaved in anhydrous HF in the presence of 10% anisole at 0 °C for 1.5 h. Crude peptides were precipitated, washed with diethyl ether, and then extracted from resin and other nonpeptide material with water. Non-cysteine-containing peptides were then frozen and lyophilized.

Cyclization. Compounds containing lactam bridges were assembled, for example, using Fmoc side chain protection of the β-amino group of Dpr and the fluorenylmethyl ester protection of the β-carboxyl of Asp, which were removed by two treatments of 20% piperidine in DMF for 10 and 15 min. Lactam formation then proceeded on the resin in the presence of 3 equiv of BOP or HBTU in NMP for 4−48 h. Completeness of coupling was monitored by Ninhydrin test, and fresh reagents were added every 4 h.

Cyclization of free sulfhydryl-containing analogues was performed following HF cleavage by adding the peptide extract to a solution of 0.07 M NH_4OAc and stirring at pH 6.8, 4 °C, for 24 h. Completion of cyclization was monitored by the Elman test and HPLC. Upon complete oxidation, peptide solution was applied to a preparative RP-HPLC column packed with C_{18}-derivatized silica gel and concentrated by eluting with 60% CH_3CN/0.1% TFA. A powder was obtained after lyophilization.

Purification and Characterization (Refer to Table 1). Crude peptides were purified by preparative reversed phase HPLC on a Waters DeltaPrep LC 3000 system equipped with a Waters 1000 Prep Pak module and a Shimadzu SPD-6A variable wavelength UV detector. The cartridges used were handpacked, in-house, with Waters poly(ethylene) sleeves and frits and Vydac C_{18} packing material (15-20 μm particle size, 30 nm pore size). The material was first eluted using a linear TEAP/60% MeCN (pH 2.25) buffer system gradient; acceptable fractions were pooled, reloaded onto the preparative cartridge, and desalted in 0.1% TFA. Purified peptides were subjected to HPLC analysis (Vydac C_{18} column, Perkin-Elmer Series 400 liquid chromatograph, Kratos Spectroflow 757 UV detector, and Hewlett-Packard Model 3390A integrator). Purity was determined by analytical HPLC using linear gradient conditions (1% B increase/min) in a solvent system comprised of A (0.1% TFA) and B (60% CH_3CN in 0.1% TFA) and a Vydac C_{18} column (5 μm particle size, 46 × 250 mm), and detection at 210 nm indicated purities similar to those determined by CZE presented in Table 1. Capillary zone electrophoresis was performed on a Beckman P/ACE System 2050 with Spectra-Physics ChromJet SP4400 integrator. Capillary was Supelco SElect P175 (50 cm × 75 μm). The buffer was 100 mM phosphoric acid adjusted to pH 2.5 by addition of 2 M sodium hydroxide. Voltage was 12 kV. Detection was at 214 nm, and the temperature was maintained at 30 °C. Injections were for 4 s from stock solution at a concentration of 0.1 mg/mL. Amino acid analysis (hydrolysis in 4 N methanesulfonic acid at 110 °C for 24 h followed by ion exchange chromatography and postcolumn derivatization with *o*-phthalaldehyde) gave the expected ratio of amino acids. LSI-MS analyses were measured with a Jeol JMS-HX110 double-focusing mass spectrometer (Jeol, Tokyo, Japan) fitted with a Cs^+ gun. Samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix. Results are presented in Table 1.

Molecular Modeling. Preliminary working models of the peptides described here were constructed by homology to the crystallographic structure of APP obtained from the Brookhaven Protein Data Bank. Hydrogen atoms were explicitly added using standard bond distances and valence angles. After interactive computer graphic analysis and manual manipulation to eliminate severe overlap (Insight II, BIOSYM Technologies), the structures were minimized with a restraining potential employed to tether the backbone atoms using DISCOVER (BIOSYM) with the CVFF force field of Hagler and co-workers.[31] Finally, the tethering potential was relaxed, and the NPY model was allowed to undergo flexible geometry minimization. Models of truncated analogues were built by deletion, mutation, and minimization of the NPY model.

Binding Assays. Receptor binding studies were performed using cultures of SK-N-MC and SK-N-BE2 human neuroblastoma whole cells (American Type Culture Collection, Bethesda, MD) as previously described.[1]

cAMP Generation. The inhibition of cAMP accumulation was measured in SK-N-MC cells. All reagents were diluted in Dulbecco's modified Eagle's medium (DME) high-glucose medium without phenol red. Cells were first washed with 500 μL of warm DME, aspirated, and then resuspended in 350–400 μL of DME. The cells were incubated with 50 μM 3-isobutyl-1-methylxanthine (IBMX) at 37 °C for 10 min prior to the addition of other drugs. NPY or analogues of interest were added at varying concentrations to the appropriate wells together with norepinephrine at a final concentration of 0.3 μM. Cells were incubated at 37 °C for 30 min. The assay was terminated by aspiration of the media followed by the addition of cold 0.1 N HCl to each well. The wells were sonicated for 5 s and the well contents transferred to test tubes. Media were evaporated overnight.

cAMP Determination. cAMP concentrations were quantified by a modified RIA using the regulatory subunit of protein kinase A as the binding protein. Samples were reconstituted in 50 mM sodium acetate solution, pH 4.5. Tritiated cAMP was diluted to a concentration of 100 000 cpm/25 μL. Various concentrations of cold cAMP were prepared as standards. Buffer and standard or sample (25 μL) followed by 25 μL of [^{3}H]cAMP trace and 25 μL of protein kinase were added to prechilled tubes on ice. Samples were vortexed and incubated at 4 °C for 60 min. At the end of the incubation period, 1 mL of charcoal slurry (20 mM KPO_4, 3.33 g/L BSA, 2.67 g/L activated charcoal, pH 6.0, with HCl) was added to each tube, and the tube was incubated at 4 °C for 15 min. The samples were centrifuged and the supernatents decanted into scintillation vials containing 10 mL of Cytoscint. Radioactivity was quantified using a Beckman Model 1600TR scintillation counter.

Change in Mean Arterial Pressure. Male Sprague–Dawley rats (250–299 g; Harlan, San Diego, CA) were used. Animals were anesthetized with urethane (1.5 g/kg). Carotide and jugular catheters constructed from PE 10 tubing were inserted for measurement of pressure and delivery of drug, respectively. Pressure measurements were made using a Gould-Stathem P23D pressure transducer and recorded on a Beckman R-611 dynograph. Arterial pressure was measured immediately prior to and 2–3 min following injection of the peptides. Mean arterial prssure was calculated as diastolic + (systolic − diastolic)/3.

Food Intake. Peptides were infused into the lateral brain ventricle at a dose of 3 nmol in 5 μL of water, where water was the vehicle control. This dose was chosen to coincide with the dose used for anxiety measurements. Food intake was measured at 1.5 and 4 h after peptide infusion. Feeding measurements were taken from preweighed dishes containing a mixture of ground rodent chow, sugar, and condensed milk (45:28:27). Rats were offered the mixture along with their regular chow 24 h prior to peptide infusion. One and one-half hours prior to infusion, regular chow was removed. Rats, however, were allowed to continue ingesting the sweetened mixture to ensure a sated state and habituation to the mixture, which was used to measure the effects of peptide infusion on food intake. Data are presented as total food ingested (g) ± SEM. Statistical analysis was calculated by one-way ANOVA and Fishers least significant difference test.

Elevated Plus Maze Model of Anxiety. The plus maze was constructed of wood and had four arms (16 in. × 3.5 in.) with a 4 in. × 4 in. hub. Two of the four arms were enclosed by 16 in. × 16 in. walls on each side, with openings at both arm and hub ends. The maze was elevated 16 in. from the floor. The remaining two open arms were covered with hardware cloth to improve footing. For the experiment, peptides or water was infused into the lateral brain ventricles over a 2 min period at a dose of 3 nmol in a 5 μL volume. Diazapam was injected intraperitoneally at a dose of 2.5 mg/kg. Sixty minutes after infusion of peptides or water vehicle, and 30 min after injection of diazapam, rats were brought into the room containing the plus maze and placed into a large open cage for 3 min. Rats were then placed onto the center of the maze, facing an open arm. Observation began immediately after the 3 min period in the open cage and continued for 5 min. The room containing the plus maze was lit with red-filtered light. Rats were required to place all four paws onto an open arm in order for an entry to be scored. Time spent in open arms was also measured, with the same entry requirement.

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