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Further Characterization of Neuropeptide Y Receptor Subtypes using Centrally Truncated Analogs of Neuropeptide Y: Evidence for Subtype-Differentiating Effects on Affinity and Intrinsic Efficacy

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SUMMARY

Previous attempts to classify neuropeptide Y receptor subtypes suffered from relying only on carboxyl-terminal analogs and fragments of neuropeptide Y. We have tested the potency and affinity of chemically different compounds, i.e., centrally truncated analogs of neuropeptide Y, in three Y_1-like (Ca^{2+} mobilization in HEL cells, blood pressure increases in pithed rats, and ^{125}I-neuropeptide Y binding in SK-N-MC cells) and two Y_2-like (^{125}I-neuropeptide Y binding to rabbit kidney membranes and presynaptic inhibition in rat vas deferens) model systems of neuropeptide Y receptors. Our data confirm the concept of two major subclasses of neuropeptide Y receptors, with some centrally truncated neuropeptide Y analogs having high affinity for Y_2-like and low affinity for Y_1-like neuropeptide Y receptors. Some of the truncated neuropeptide Y analogs are antagonists at Y_1-like receptors and (possibly partial) agonists at Y_2-like receptors. Our data also indicate that amino acid residues distal from the amino- and carboxyl-terminal ends of the peptide may subtype-selectively affect affinity and intrinsic efficacy of peptide agonists at neuropeptide Y receptors.

NPY is a putative neurotransmitter that is found in many central and peripheral neurons and may be involved in the neuronal control of numerous physiological processes, including cardiovascular regulation and food intake (1–3). NPY elicits its effects via specific receptors, which apparently belong to the superfamily of heptahelical guanine nucleotide-binding protein-coupled receptors (4). Five years after the discovery of NPY, Wahlestedt *et al.* (5), proposed the existence of NPY receptor subtypes, which were termed Y_1 and Y_2. This proposal has been supported by demonstration of an inverse order of potency in physiological assays and of affinity in binding studies for [Pro^{34}]-substituted analogs of NPY ([Leu^{31},Pro^{34}]NPY and [Pro^{34}]NPY) and carboxyl-terminal fragments of NPY (NPY-$_{13-36}$ and NPY-$_{18-36}$), with the former being selective for the Y_1 and the latter for the Y_2 subtype (6–9). Although the related peptide PYY activates both Y_1 and Y_2 subtypes of NPY receptors, PYY does not mimic NPY responses in some tissues, indicating the existence of a third PYY-insensitive NPY receptor subtype (Y_3); some data even indicate that additional subtypes may exist (4).

An exact pharmacological definition of NPY receptor subtypes, however, has been hampered by two obstacles. First, present attempts at NPY receptor subtype classification are based solely on agonists, because appropriate antagonists are not available (4). Second, all tools for the subclassification of NPY receptor subtypes are of limited chemical diversity, i.e., carboxyl-terminal analogs and fragments of NPY. Because classical pharmacological definition of receptor subtypes requires selective antagonists and compounds with considerably different chemical structures, present attempts to classify NPY receptor subtypes have to be considered tentative.

Recently, centrally truncated NPY analogs have been introduced that can potently interact with some NPY receptors (10–13). These analogs of NPY have been centrally truncated and the PP fold has been substituted by an Aca or 8-aminooctanoic acid spacer. In the present study, we have compared the potency of such compounds with that of NPY, carboxyl-terminal frag-

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ABBREVIATIONS: NPY, neuropeptide Y; PP, pancreatic polypeptide; PYY, peptide YY; Aca, ϵ-aminocaproic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ments of NPY, and [Pro^{34}]-substituted analogs of NPY in various assay systems of Y_1- and Y_2-like NPY receptors.

Materials and Methods

Ca^{2+} experiments. HEL cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, at a density of 4–8 × 10^5 cells/ml. The free intracellular Ca^{2+} concentration was determined as previously described, using the fluorescent indicator fura-2 and the dual-wavelength ratio method (14). In the experiments on agonistic effects, fluorescence was monitored until a peak had been reached or (if no peak was observed) for a minimum of 4 min (NPY-stimulated fluorescence changes peaked within 40 sec) (14, 15). Because the absolute response to NPY varied among experiments, the Ca^{2+} increase in respose to 100 nM NPY was determined in each set of cuvettes, and data were expressed as percentage of that response (9); the Ca^{2+} increase elicited by 100 nM NPY ranged between 50 and 200 nM.

In the experiments on antagonistic effects, base-line fluorescence was monitored for 30 sec, analogs were added, and NPY was added another 30 sec later; fluorescence was monitored until a peak had been reached. The potency and efficacy of NPY in the absence and presence of its centrally truncated analogs were calculated from concentration-response curves, with five concentrations of NPY, by fitting the experimental data to a sigmoidal curve with a Hill slope of 1, using the InPlot program (GraphPAD Software, San Diego, CA). The −log EC_{50} (pEC_{50}) of NPY in the presence of the analogs was compared with the pEC_{50} in the absence of the analogs found in paired experiments. Statistical significance of effects of the semipeptide analogs of NPY was assessed by one-way analysis of variance; if the variance between groups was found to be significantly greater than that within groups, two-tailed *t* tests with Bonferroni corrections for multiple comparisons were performed. If the difference was statistically significant in the Bonferroni *t* test, a pK_b value was calculated for each experiment and the individual pK_b values were averaged; pK_b values were calculated according to the equation $pK_b = \log[B] - \log(DR - 1)$, with $[B]$ being the molar concentration of the antagonist and DR the ratio of NPY EC_{50} values in the presence and absence of the antagonist.

Blood pressure measurements. The blood pressure-increasing effect of NPY and its analogs was tested in male rats (strain Chbb:THOM; ≈280 g) that had been anesthetized with 60 mg/kg sodium pentobarbital (Nembutal), intraperitoneally, artificially respirated, and pithed according to the method of Gillespie and Muir (16). Peptides were injected into the jugular vein, in a volume of 100 μl/100 g of body weight, and the dose of peptide that increased mean arterial pressure by 30 mm Hg (ED_{30}) was determined as previously described (10). Each compound was tested in at least three animals.

Radioligand binding in SK-N-MC cells. SK-N-MC human neuroblastoma cells were grown in minimum essential medium supplemented with 4 mM glutamine, 10% fetal bovine serum, 1% nonessential amino acids, and 1 mM sodium pyruvate, in a humidified incubator with an atmosphere of 95% air and 5% CO_2, at 37°. For the binding assay, cells were harvested and resuspended at a density of ≈2.5 × 10^5/ml (final concentration), in assay buffer consisting of minimum essential medium supplemented with 25 mM HEPES, 0.5% bovine serum albumin, 50 μM phenylmethylsulfonyl fluoride, 0.1% bacitracin, and 3.75 mM $CaCl_2$. The binding reaction was started by addition of the cell suspension to the assay buffer containing 30 pM ^{125}I-NPY; nonspecific binding was defined as that in the presence of 1 μM unlabeled NPY. After 3 hr of incubation under constant agitation at room temperature, the cells were centrifuged at 4°. The supernatant was drawn off, and the pellets were washed by centrifugation in phosphate-buffered saline. The remaining radioactivity in the final pellet was determined in a scintillation counter. Under these conditions, the K_d for ^{125}I-NPY was approximately 0.3 nM. Competition binding curves were analyzed by fitting the experimental data to a sigmoidal curve, using the InPlot program. Because the pooled data from all experiments were fitted, only mean K_i values, without standard errors, are given below.

Radioligand binding in rabbit kidney. Radioligand binding to rabbit kidney cortical membranes was measured according to the method of Chang *et al.* (17), with modifications. Briefly, kidneys were obtained from adult male chinchilla-bastard rabbits (strain Chbb:CH), and renal cortex was homogenized with a Polytron, in 5 volumes of ice-cold Tris·HCl (50 mM, pH 7.4). The homogenate was centrifuged for 5 min at 1000 × *g*, and the supernatant was centrifuged for 25 min at 40,000 × *g* three times. The final pellet was resuspended in 125 volumes of Tris·HCl (50 mM, pH 7.4) supplemented with 5 mM $MgCl_2$, 0.1 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine serum albumin, and 0.25 mg/ml bacitracin. Aliquots of the membrane suspension (≈25 μg of protein) were added to tubes containing ^{125}I-NPY (≈12 pM), in a final volume of 250 μl; some tubes contained an additional 10 nM unlabeled NPY, for determination of nonspecific binding. After 60 min of incubation at room temperature, the reaction was terminated by washing the membranes twice, by 15-min centrifugations at 1500 × *g*; the radioactivity remaining in the pellets was determined in a LKB γ counter. Under these conditions, nonspecific binding was approximately 10–20% of total binding, and the K_d for ^{125}I-NPY was approximately 150 pM. Competition binding curves were analyzed by fitting the experimental data to a sigmoidal curve, using the InPlot program. Because the pooled data from all experiments were fitted, only mean K_i values, without standard errors, are given below.

Presynaptic inhibition. Inhibition of the field stimulation-induced (15 V, 1-msec duration, 0.15 Hz) twitch response was determined in seminal ducts from rat (strain Chbb:THOM; ≈250 g) vas deferens, as previously described (10). Concentration-response curves were established for each compound, and the concentration that reduced vas deferens contraction by 50% (EC_{50}) was determined graphically. Due to the large amounts of peptide required in these experiments, only two preparations were tested for some peptides.

Chemicals. Centrally truncated analogs of NPY were synthesized as previously described (10, 13, 18), and their structures are depicted in Fig. 1. For convenience and brevity, we use the following designations throughout the manuscript: **I**, NPY-1–4-Aca-25–36; **II**, [Gly^2]NPY-1–4-Aca-25–36; **III**, [Ala^{28}]NPY-1–4-Aca-25–36; **IV**, 1,2,3,4-tetrahydro-2-naphtylbutyl-NPY-25–36; and **V**, [Phe^{27,36},Tyr^{30}]NPY-1–4-Aca-25–36. NPY, NPY-$_{13-36}$, NPY-$_{18-36}$, and [Leu^{31},Pro^{34}]NPY were obtained from Bissendorf (Hannover, Germany).

Data presentation. Data are shown as mean ± standard error of *n* experiments. Unless otherwise indicated, the three-letter code for amino acids is used throughout the manuscript.

compound	chemical structure
I	Y^1PSK-Aca-R^{25}HYINLITRQRY^{36}
II	Y^1<u>G</u>SK-Aca-R^{25}HYINLITRQRY^{36}
III	Y^1PSK-Aca-R^{25}HY<u>A</u>NLITRQRY^{36}
IV	1,2,3,4-tetrahydro-2-naphtylbutyl-R^{25}HYINLITRQRY^{36}
V	Y^1PSK-Aca-R^{25}H<u>F</u>IN<u>Y</u>ITRQR<u>F</u>^{36}

Fig. 1. Chemical structures of centrally truncated NPY analogs. Structures are depicted in the single-letter amino acid code. Amino acid substitutions relative to native NPY are *underlined*; amino acid positions in the native NPY are indicated by *superscript*.

Results

Ca^{2+} mobilization in HEL cells. We have previously demonstrated that [Pro^{34}]NPY is a full agonist and NPY-$_{13-36}$ and NPY-$_{18-36}$ are partial agonists for mobilizing Ca^{2+} in HEL cells (9, 14). At concentrations up to 10 μM (i.e., ≈2000 times the EC_{50} for NPY), none of the centrally truncated NPY analogs had significant agonistic effects at the HEL cell NPY receptor (Table 1). Compound **IV** (10 μM) produced a Ca^{2+} increase that was 176% of that elicited by a maximally effective NPY concentration (100 nM); because we did not observe any effect of this compound at 1 or 3 μM and because its effect at 10 μM considerably exceeded the maximal NPY effect, we believe that this is a nonspecific effect not mediated via NPY receptors and possibly due to the amphiphilic (possibly detergent) properties of this peptide.

Because the lack of agonistic effects could come from low affinity or from weak efficacy, we constructed concentration-response curves for NPY in the absence and in the presence of 1 μM levels of the analogs. The analogs **I**, **II**, and **V** did not significantly shift the concentrations-response curve for NPY (Table 1), suggesting that their affinity at the HEL cell NPY receptor is considerably less than 1 μM. In contrast, compounds **III** and **IV** significantly shifted the NPY concentration-response curve to the right, without significant alterations of its maximum (Table 1; Fig. 2). From these rightward shifts, we calculated $-\log K_b$ (pK_b) values of 6.33 ± 0.10 and 6.66 ± 0.07 for analogs **III** and **IV**, respectively. These submicromolar affinities, together with the lack of agonistic effects on NPY receptors at concentrations up to 10 μM, suggest that compounds **III** and **IV** are antagonists at the HEL cell Y_1-like NPY receptor, with very little, if any, intrinsic efficacy.

Radioligand binding in SK-N-MC cells. In confirmation of previous results (7, 19), NPY and [Leu^{31},Pro^{34}]NPY had high affinity (0.45 and 0.73 nM, respectively), whereas NPY-$_{13-36}$ and NPY-$_{18-36}$ had low affinity (709 and >100 nM, respectively), at SK-N-MC cell [^{125}I]NPY binding sites (Table 2). For the centrally truncated NPY analogs, the following K_i values were obtained: analog **I**, 4545 nM; analog **II**, 10,909 nM; analog **III**, 33,636 nM; analog **IV**, 909 nM; and analog **V**, 9091 nM (Table 2). It should be noted that the affinities of compounds **III** and **IV** are lower than their apparent affinities in HEL cells (see above); on the other hand, we (14) and others (20) have previously observed that binding affinities at NPY receptors are consistently lower than functional affinity estimates for various peptide and nonpeptide antagonists, possibly due to the agonistic properties of the radioligand used.

Blood pressure increases in pithed rats. [Leu^{31},Pro^{34}]NPY increased mean arterial pressure in pithed rats with a potency that was similar to that of NPY (Fig. 3; Table 2). In contrast, NPY-$_{13-36}$ and NPY-$_{18-36}$ were 392- and 1083-fold less potent, respectively, than NPY (Fig. 3; Table 2). Thus, the increase in systolic blood pressure is mediated by a Y_1-like

TABLE 1

Agonistic and antagonistic effects of centrally truncated NPY analogs on Ca^{2+} mobilization in HEL cells

A shows the response to the analogs in the indicated concentrations. B shows the parameters of NPY concentration-response curves obtained in the absence and presence of 1 μM levels of the analogs. Ca^{2+} increases are expressed as percentage of the response to 100 nM NPY and are mean ± standard error of three experiments.

A. Agonistic effects	Analog response at	
	1 μM	10 μM
	%	
Compound **I**	−3 ± 3	−12 ± 16
Compound **II**	1 ± 1	0 ± 1
Compound **III**	−6 ± 6	−9 ± 7
Compound **IV**	−1 ± 1	176 ± 66
Compound **V**	1 ± 1	7 ± 5

B. Antagonistic effects	NPY response	
	pEC_{50}	Maximum
		%
Control	8.40 ± 0.05	108 ± 2
+**I**	8.28 ± 0.14	115 ± 7
+**II**	8.48 ± 0.06	107 ± 9
+**III**	7.85 ± 0.12[a]	129 ± 8
+**IV**	7.61 ± 0.11[a]	116 ± 10
+**V**	8.23 ± 0.13	119 ± 11

[a] $p < 0.05$ versus the respective paired NPY concentration-response curve in the absence of the analog, in a paired two-tailed t test.

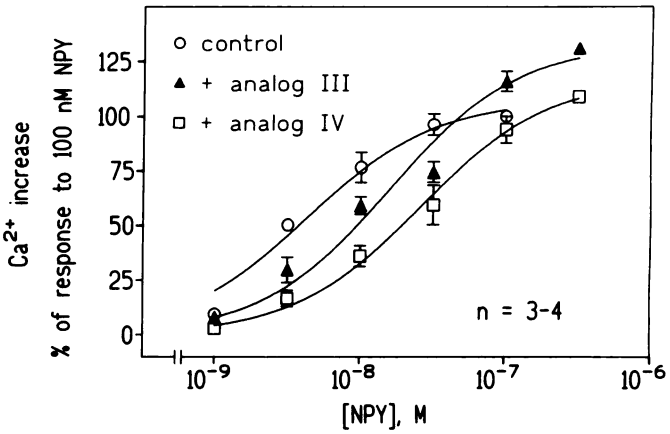

Fig. 2. Effect of analogs **III** and **IV** on NPY-stimulated Ca^{2+} mobilization in HEL cells. NPY concentration-response curves were obtained in the absence (○) or presence of 1 μM analog **III** (▲) or analog **IV** (□). Data are mean ± standard error, after normalization of Ca^{2+} increases to those elicited by 100 nM in a paired cuvette. The mean pK_b values for analogs **III** and **IV** were 6.33 ± 0.10 and 6.66 ± 0.07, respectively.

TABLE 2

Comparison of relative equieffective concentrations and doses of NPY analogs in five model systems

All data are expressed as concentration/dose of analog required relative to that of NPY. In order to obtain such ratios, we used EC_{50} values (agonists) and K_b values (partial agonists and antagonists) for Ca^{2+} mobilization in HEL cells, the dose of peptide that elevates mean arterial pressure by 30 mm of Hg, the K_i values obtained in radioligand binding to SK-N-MC cells and rabbit kidney membranes, and the concentration of peptide that inhibits field stimulation-induced contraction of rat vas deferens by 50%. The respective reference values for NPY are as follows: EC_{50} in HEL cells, 5.0 nM; ED_{30} in the pithed rat, 0.1 nmol/kg; K_i in SK-N-MC cells, 0.45 nM; K_i in rabbit kidney, 0.46 nM; and EC_{50} in rat vas deferens, 19 nM. [Pro^{34}]NPY was used instead of [Leu^{31},Pro^{34}]NPY in HEL cells; HEL cell data for [Pro^{34}]NPY, NPY-$_{13-36}$, and NPY-$_{18-36}$ are taken from Ref. 14.

	Relative effective concentration				
	Ca^{2+} mobilization in HEL cells	^{125}I-NPY binding in SK-N-MC cells	Blood pressure increase in pithed rat	^{125}I-NPY binding in rabbit kidney	Presynaptic inhibition in rat vas deferens
[$Leu^{31}Pro^{34}$]NPY	1.2	1.6	3.9	100	168
NPY-$_{13-36}$	100	1,560	392	1.4	4
NPY-$_{18-36}$	59	>200	1,083	6.6	111
Compound **I**	ND[a]	10,000	692	7.0	9
Compound **II**	ND	24,000	1,000	11	74
Compound **III**	94	74,000	>4,600	56	526
Compound **IV**	44	2,000	>10,000	60	526
Compound **V**	ND	20,000	>2,600	13	74

[a] ND, not determined.

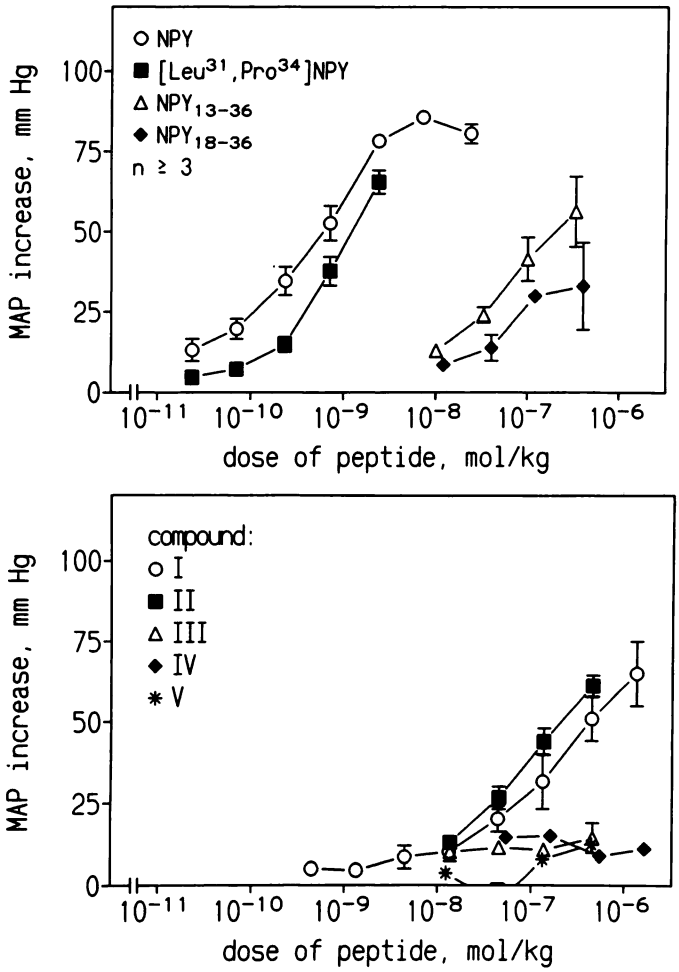

Fig. 3. Effects of NPY and its peptide and semipeptide analogs on mean arterial pressure (*MAP*) in pithed rats. Data are mean ± standard error of mean arterial pressure increases produced by NPY and its carboxyl-terminal analogs and fragments (*upper*) or by the centrally truncated NPY analogs (*lower*). *Abscissa*, dose of peptide (mol/kg of body weight) injected i.v.

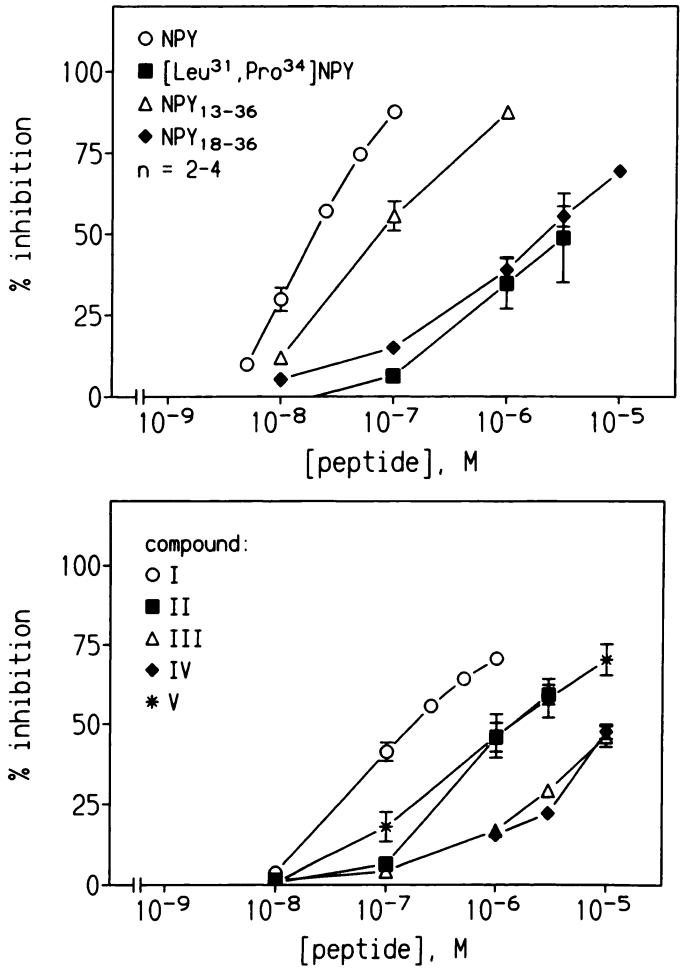

Fig. 4. Effects of NPY and its peptide and semipeptide analogs on the field stimulation-induced contraction of rat vas deferens. Data are mean ± standard error.

NPY receptor. In confirmation of previous reports (21, 22), some of these peptides lowered blood pressure when doses were increased further (data not shown).

The centrally truncated analogs **I** and **II** had similarly weak effects on blood pressure as did the carboxyl-terminal fragments, compared with NPY; the analogs **III**, **IV**, and **V** did not increase blood pressure appreciably, which is in good agreement with their low affinity (analog **V**) and/or antagonistic properties (analogs **III** and **IV**) (Fig. 3; Table 2).

Radioligand binding in rabbit kidney. NPY and all of its analogs were evaluated in competition binding studies and yielded the following K_i values: NPY, 0.46 nM; [Leu^{31},Pro^{34}]NPY, 46.2 nM; NPY-$_{13-36}$, 0.62 nM; NPY-$_{18-36}$, 3.05 nM; analog **I**, 3.23 nM; analog **II**, 5.17 nM; analog **III**, 25.7 nM; analog **IV**, 28.1 nM; and analog **V**, 5.82 nM (Table 2).

Presynaptic inhibition in rat vas deferens. NPY-$_{13-36}$ inhibited field stimulation-induced contraction of rat vas deferens only 4-fold less potently than did NPY, whereas [Leu^{31},Pro^{34}]NPY was 168-fold less potent (Fig. 4; Table 2), demonstrating mediation via a Y_2-like NPY receptor subtype. Although NPY-$_{18-36}$ was somewhat more potent than [Leu^{31},Pro^{34}]NPY in this system, it was considerably weaker than NPY-$_{13-36}$ (Fig. 4; Table 2), despite its high affinity for the Y_2-like binding sites.

Among the centrally truncated analogs, compound **I** was similar in potency to NPY-$_{13-36}$, and compounds **II** and **V** were similar in potency to NPY-$_{18-36}$ (Fig. 4; Table 2). The analogs **III** and **IV** had the lowest potency of all tested compounds (Fig. 4; Table 2), although they exhibited moderate affinity in rabbit kidney.

Discussion

Growing evidence suggests that NPY mediates its numerous physiological effects via at least two subtypes, which have been designated Y_1 and Y_2 (4, 8). Additionally, a NPY receptor subtype may exist that is insensitive to the related PYY (in contrast to Y_1- and Y_2-like receptors) and has tentatively been designated Y_3 (4); a Y_3-like NPY receptor may have recently been cloned (23). Although the existence of NPY receptor subtypes is generally accepted, their exact pharmacological definition remains troublesome, because it presently relies on agonists of a single chemical class only. Agonist potency, however, is unreliable for receptor classification, because it is a composite parameter of affinity, intrinsic efficacy, receptor reserve, and receptor accessibility (24), and present knowledge on all of these factors is incomplete. Receptor accessibility for

the fairly large and sticky NPY molecule may be limited in many cases, as indicated by EC_{50} values in some model system, which are considerably higher than NPY affinity for its receptors (25). Although recent studies have suggested that there is no receptor reserve for NPY-stimulated Ca^{2+} mobilization in HEL cells (14), similar information is lacking regarding other model systems. The intrinsic efficacy of many agonists at NPY receptors remains unclear, but it has been demonstrated that the observed efficacy may differ among NPY receptor subtypes. Thus, NPY-$_{18-36}$ appears to be a full agonist at Y_2-like receptors (9, 25), a partial agonist at Y_1-like receptors (9), and a pure antagonist at Y_3-like receptors (20). Present affinity estimates in binding studies rely on the agonist ligand NPY, which may selectively label certain states of the receptor and whose binding parameters may be subject to alterations, e.g., changes in the cellular content of guanine nucleotide-binding proteins (19). Thus, it has been shown that functional estimates of the affinity of partial agonists and pure antagonists (pK_b and pA_2 values) may differ by >10-fold from binding affinities (pK_i values) obtained in the same model system (14, 20). Finally, it should be noted that NPY action may be complex and involve not only direct receptor activation but also indirect mechanisms, such as presynaptic inhibition, postsynaptic potentiation, and histamine release, which may be mediated by distinct receptor subtypes or occur nonspecifically (4). This complexity necessitates the use of chemically diverse compounds to unequivocally establish the pharmacological characteristics of NPY receptor subtypes.

Because previous attempts to classify NPY receptor subtypes relied on chemically similar compounds, i.e., carboxyl-terminal fragments and analogs of NPY, the present study has tested the effects of several NPY analogs, which are centrally truncated, in five model systems for NPY receptors. Because the absolute potency of NPY itself varied considerably among these models, we have used ratios of equieffective concentrations/doses of analog, relative to those for NPY, to compare them. For comparison, we have also tested the established Y_1-selective [Leu^{31},Pro^{34}]NPY and Y_2-selective NPY-$_{13-36}$ and NPY-$_{18-36}$. Our results demonstrate that the NPY receptors mediating Ca^{2+} mobilization in HEL cells and blood pressure increases in pithed rats and the binding sites in SK-N-MC cells belong to the Y_1-like subtype, whereas the binding sites present in rabbit kidney and the receptors mediating presynaptic inhibition in rat vas deferens belong to the Y_2-like subtype. Similar classification of these receptors has been proposed previously (7, 9, 18, 19, 25–30). It should be noted that the agonistic potency of NPY-$_{13-36}$ and NPY-$_{18-36}$, relative to that of NPY, in the blood pressure and the vas deferens assays is lower than their antagonistic potency or binding affinity at the respective subtypes; this may be related to smaller intrinsic efficacy than that of NPY, which has previously been demonstrated (9, 14). A summarization of the selectivity profile of the aforementioned peptides and the centrally truncated analogs is given in Fig. 5.

The analogs **I**, **II**, and **V** had much lower affinities at the Y_1-like receptors in HEL and SK-N-MC cells than at the Y_2-like receptor in rabbit kidney. Similar conclusions can be drawn indirectly from previously published data (12), which demonstrate a relatively low affinity of analog **I** for [^{3}H]propionyl-NPY binding sites in rat cerebral cortex, because studies in our

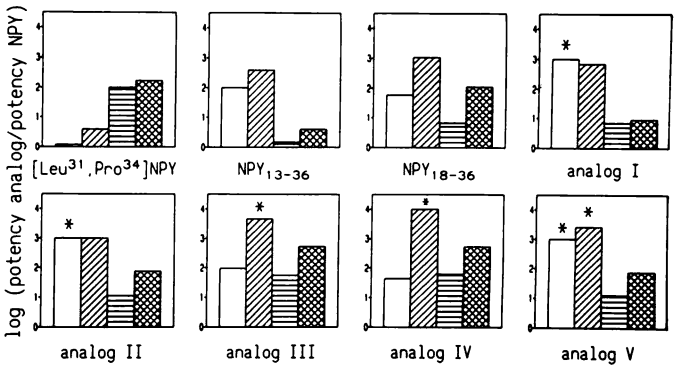

Fig. 5. Comparative potency of NPY analogs in four model systems. The ratio log(potency of analog/potency of NPY) was determined for Ca^{2+} mobilization in HEL cells (□), blood pressure increases in pithed rats (▨), rabbit kidney ^{125}I-NPY binding sites (▤), and presynaptic inhibition in rat vas deferens (■). A numeric representation of these values is given in Table 2. *, Value is greater than or equal to the one shown (an exact ratio could not be determined, due to lack of agonistic effects at the available peptide amounts).

laboratory[1] and other laboratories (30) suggest that rat cerebral cortex contains predominantly Y_1-like NPY receptors. Accordingly, the analogs **I**, **II**, and **V** exhibited high to moderate potency in the Y_2-like functional assay in vas deferens but low to undetectable potency in the Y_1-like blood pressure assay. Thus, centrally truncated NPY analogs with and without amino- or carboxyl-terminal amino acid substitutions can discriminate NPY receptor subtypes and thereby confirm the Y_1/Y_2 concept, although their chemistry differs considerably from the previously used carboxyl-terminal fragments and analogs. The selectivity for Y_2 receptors appeared to be greatest for analog **I** and was intermediate between those of NPY-$_{13-36}$ and NPY-$_{18-36}$.

In contrast, analogs **III** (which is identical to analog **I** except for an alanine instead of an isoleucine in position 28) and **IV** (which contains an aromatic spacer linked to Arg^{25} of the 12 carboxyl-terminal amino acids of NPY) showed little selectivity for the Y_1-like HEL or SK-N-MC cell receptor versus the Y_2-like rabbit kidney NPY receptor, having moderate affinity for both. Despite their moderate Y_1 and Y_2 affinity, analogs **III** and **IV** lacked agonistic effects in the HEL cell and the blood pressure assays and had a low potency in rat vas deferens. This discrepancy indicates low intrinsic efficacy. This idea is supported by the observation that both analogs shifted the NPY concentration-response curves in HEL cells to the right, without significantly altering the maximal NPY effect. Thus, analogs **III** and **IV** may be antagonists at Y_1-like receptors and partial agonists at Y_2-like receptors. This conclusion remains tentative, however, because the available amounts of peptide did not allow us to directly determine antagonistic properties of analogs **III** and **IV** in the blood pressure and the vas deferens assays. Thus, our data confirm the concept of Y_1- and Y_2-like receptors, using NPY analogs that differ considerably in their chemical structures from the carboxyl-terminal fragments and analogs of NPY used previously; however, not all centrally truncated NPY analogs exhibit this subtype selectivity. On the other hand, our data provide initial evidence for even further heterogeneity, because NPY-$_{13-36}$ and NPY-$_{18-36}$ differed consid-

[1] F. Feth, K. Lewejohann, W. Rascher, and M. C. Michel, unpublished observations.

erably in their potency in the rat vas deferens but not in the rabbit kidney assay and because analogs **III** and **IV** had similar affinities (determined functionally) in HEL cells but not in SK-N-MC cells (determined by radioligand binding), although the respective model systems are thought to represent the same subtype of NPY receptors.

The present data also expand our understanding of the interaction of NPY with its receptors in other ways. Based on the crystal structure of PP, with which NPY shares considerable sequence homology, it has been proposed that the secondary structure of NPY comprises a polyproline helix that lies antiparallel to an amphiphilic α-helix (10, 13, 31). According to this putative three-dimensional structure, it has been proposed that the amino terminus of NPY adjoins the six carboxyl-terminal amino acids to form the receptor-binding domain of the peptide (31). This concept has been supported by previous reports in which centrally truncated semipeptide analogs of NPY retained considerable affinity for an NPY receptor (10, 11, 32). On the other hand, it has been reported that perturbation of the PP fold by substitution with helix-breaking proline residues results in loss of affinity at Y_1- and Y_2-like NPY receptors (9, 33). Similarly, it has been proposed that the amphiphilic structure generated by the antiparallel polyproline and the α-helix may serve important purposes in presenting NPY to its receptors in a correct conformation (34). The present data demonstrate that substitution of the PP fold by an organic spacer has considerably greater effects on the affinity and efficacy at Y_1-like than at Y_2-like NPY receptors. Thus, either centrally truncated analogs are highly Y_2-selective (compounds **I, II,** and **V**) or, at least, their efficacy at Y_2 receptors appears to be greater than that at Y_1 receptors (compounds **III** and **IV**). These data suggest that not only amino- and carboxyl-terminal amino acids but also the PP fold affect affinity and intrinsic efficacy at NPY receptors in a subtype-selective manner.

In summary, our data confirm the Y_1/Y_2 concept of NPY receptor subtypes by using centrally truncated NPY analogs, which preferentially stimulate Y_2-like receptors due to selectivity in affinity and/or intrinsic efficacy. Some of these compounds appear to be antagonists at Y_1-like receptors and may be partial agonists at Y_2-like receptors. Our data also suggest that the PP fold may be involved in the subtype-selective determination of affinity and intrinsic efficacy.

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