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[Leu^{31},Pro^{34}]Neuropeptide Y: A specific Y_1 receptor agonist

(peptide receptors/cytoplasmic free calcium/vasoactive peptides/blood pressure)

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ABSTRACT **Two types of binding sites have previously been described for 36-amino acid neuropeptide Y (NPY), called Y_1 and Y_2 receptors. Y_2 receptors can bind long C-terminal fragments of NPY—e.g., NPY-(13–36)-peptide. In contrast, Y_1 receptors have until now only been characterized as NPY receptors that do not bind such fragments. In the present study an NPY analog is presented, [Leu^{31},Pro^{34}]NPY, which in a series of human neuroblastoma cell lines and on rat PC-12 cells can displace radiolabeled NPY only from cells that express Y_1 receptors and not from those expressing Y_2 receptors. The radiolabeled analog, [^{125}I-Tyr^{36}]monoiodo-[Leu^{31},Pro^{34}]NPY, also binds specifically only to cells with Y_1 receptors. The binding of this analog to Y_1 receptors on human neuroblastoma cells is associated with a transient increase in cytoplasmic free calcium concentrations similar to the response observed with NPY. [Leu^{31},Pro^{34}]NPY is also active *in vivo* as it is even more potent than NPY in increasing blood pressure in anesthetized rats. It is concluded that [Leu^{31}, Pro^{34}]NPY is a specific Y_1 receptor agonist and that the analog or variants of it can be useful in delineating the physiological importance of Y_1 receptors.**

Neuropeptide Y (NPY) is a major regulatory peptide both in the central and peripheral nervous system (1). Centrally, NPY is believed to be involved especially in the regulation of food intake, memory processing, and circadian rhythm. In the peripheral nervous system, NPY functions mainly as a transmitter in sympathetic nerves, where it acts together with norepinephrine in the regulation of vascular tone. (The neuroanatomy and physiology of NPY was recently reviewed in a symposium volume; ref. 2.)

The primary structure of NPY has been well conserved during evolution (3, 4). The 36-amino acid peptide belongs to the pancreatic polypeptide-fold (PP-fold) family of peptides, which are characterized by a common tertiary structure, the so-called PP-fold (5). In addition to NPY, a pancreatic hormone [pancreatic polypeptide (PP)] and an intestinal hormone [peptide YY (PYY)] belong to the family (6, 7). The PP-fold structure has been characterized in great detail (<1 Å) by x-ray diffraction analysis of crystals of avian PP (8). The structure consists of two antiparallel helices, an N-terminal polyproline helix, and a long amphiphilic α-helix, which are held together by a core of interdigitating hydrophobic residues (Fig. 1) (5). An intriguing feature of these peptides is that the PP-fold, despite the lack of stabilizing covalent bridges, appears to be stable in aqueous solution as indicated by, for instance, circular dichroism studies (5, 8, 9).

Specific binding sites for NPY were initially described in membrane preparations from brain tissue (10–12) but also have been characterized recently in peripheral tissues (13, 14), on dorsal root ganglionic cells (15), and on different

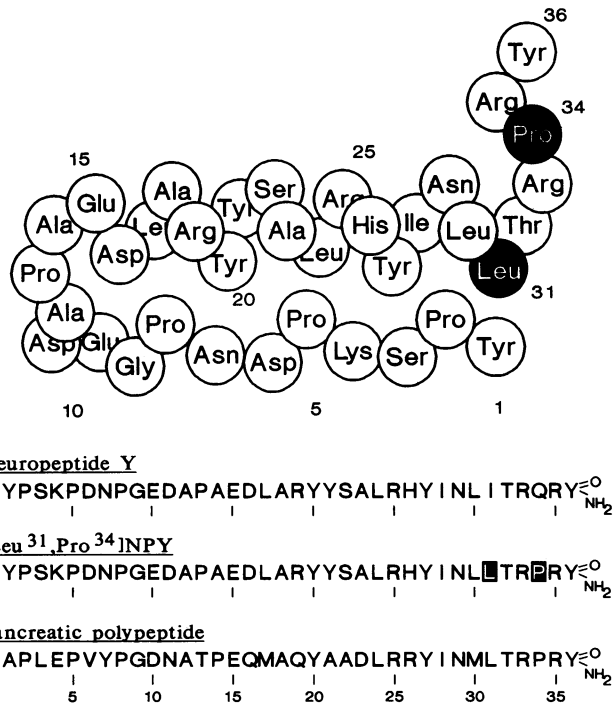

FIG. 1. Diagram of the structure of [Leu^{31},Pro^{34}]NPY and the aligned sequences of NPY, [Leu^{31},Pro^{34}]NPY, and PP. The schematic structure of the NPY analog shown at the top was based on the x-ray structure of avian PP (5).

neuronal cell lines (16, 17). It has been suggested that the binding sites for NPY occur in two separate forms, designated Y_1 and Y_2 receptors (4, 16–19). The distinction between these receptor types was based on the binding and effects of long C-terminal fragments of NPY, especially the fragment 13–36, which only bound to Y_2 receptors. Postsynaptic NPY receptors are apparently of both the Y_1 and the Y_2 types, whereas the presynaptic receptors are mainly of the Y_2 type (4). The Y_2 receptor is also the major receptor type in the central nervous system. The C-terminal segment is very similar among PP-fold peptides, and the C-terminal amide function is essential for their biological activity (18, 20). Previously, we have suggested that residue number 34, which is glutamine in NPY and proline in PP (Fig. 1), is one of the major structural elements that determines whether a particular peptide is recognized by one or the other type of receptor (4). As part of a structure–function study on the three-

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Abbreviations: NPY, neuropeptide Y; PP, pancreatic polypeptide; $[Ca^{2+}]_i$, cytoplasmic calcium concentrations.

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dimensional structure of PP-fold peptides, we synthesized analogs of NYP and PP in which the C-terminal segment of one of the peptides was combined with the PP-fold of the other. The first analog, which contained the C-terminal hexapeptide of NPY and the PP-fold of PP, [Ile^{31},Gln^{34}]PP, reacted poorly with PP receptors but was a full agonist on NPY Y_2 receptors, which supported the working hypothesis (J.F., N.L.-J., S.G.M., H.T., and T.W.S., unpublished data). The "reverse" analog in which the C-terminal hexapeptide of PP was combined with the PP-fold of NPY, [Leu^{31},Pro^{34}]NPY (Fig. 1), did not react with NPY Y_2 receptors but showed increased (although still poor) reaction with PP receptors. Most important, [Leu^{31},Pro^{34}]NPY was found to be a specific high-affinity ligand for Y_1 receptors. In the present paper we describe the receptor binding characteristics of [Leu^{31},Pro^{34}]NPY together with data on its effect on intracellular calcium concentrations ($[Ca^{2+}]_i$) and its effect on blood pressure *in vivo*.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. *Reagents.* *tert*-Butyloxycarbonyl amino acid derivatives were purchased from Bachem. The *p*-methylbenzhydrylamin resin (0.38 mmol of amine per g) was from Peptide Institute (Osaka), and dicyclohexylcarbodiimide and 1-hydroxybenzotriazole were from Fluka. All solvents were from Merck. Reagents were of analytical or HPLC grade.

Synthesis. The peptides [Leu^{31},Pro^{34}]NPY and NPY were synthesized by automatic stepwise solid-phase synthesis on an Applied Biosystems model 430-A peptide synthesizer by using the small-scale *tert*-butyloxycarbonyl chemistry (1 mmol of amino acid per 0.1 mmol of peptidyl resin) according to the manufacturer's manual. The coupling was tested after each step by the quantitative ninhydrin assay (21, 22); the average coupling yields were 98.7% and 99.3% for NPY and [Leu^{31},Pro^{34}]NPY, respectively. Side chain-protecting groups were as follows: 2-bromobenzyloxycarbonyl for tyrosine, 4-toluenesulfonyl for arginine, 2-chlorobenzyloxycarbonyl for lysine, benzyloxymethyl for histidine, and benzyl ester for aspartic and glutamic acids, except for aspartic acid-6 (cyclohexyl ester). The peptides were cleaved from the resin by hydrogen fluoride as described (23); the overall yield was 20.1% and 41.5% for NYP and [Leu^{31},Pro^{34}]NPY, respectively.

Purification. After cleavage the concentrated peptides were dissolved in 50% acetic acid, diluted 1:5 in water, purified by gel filtration on a G-25 superfine column, eluted with 2% acetic acid/15% (vol/vol) isopropanol, freeze-dried, and purified by reverse-phase HPLC. A Vydac C_{18} column (1.6 × 20 cm) was eluted with a flow of 6 ml/min and a gradient of acetonitrile from 30% to 45% in 0.2 M ammonium acetate (pH 3.5) over 45 min. Amino acid analysis of the purified peptides after acid hydrolysis gave the following compositions, expressed in molar ratios, which are in good agreement with the theoretically expected values (shown in parentheses): [Leu^{31},Pro^{34}]NPY, $Asx_{5.0(5)}Thr_{0.9(1)}Ser_{1.8(2)}Glx_{2.0(2)}Pro_{4.9(5)}Gly_{0.9(1)}Ala_{4.0(4)}Ile_{0.9(1)}Leu_{4.0(4)}Tyr_{4.7(5)}Lys_{1.0(1)}His_{1.0(1)}Arg_{4.4(4)}$; and NPY, $Asx_{5.0(5)}Thr_{0.9(1)}Ser_{1.8(2)}Glx_{2.9(3)}Pro_{3.8(4)}Gly_{1.0(1)}Ala_{4.1(4)}Ile_{2.0(2)}Leu_{3.0(3)}Tyr_{4.6(5)}Lys_{1.0(1)}His_{1.0(1)}Arg_{4.4(4)}$. The integrity of the C-terminal amide function was monitored by a radioimmunoassay that is totally specific for this modification in NPY using NPY antibody 8999 and [^{125}I-Tyr^1]monoiodo-NPY (ref. 17 and unpublished observations). The circular dichroic spectra of [Leu^{31},Pro^{34}]NPY and authentic NPY were very similar, indicating no major difference in the secondary structure of the analog as compared to NPY (data not shown).

Iodination of Peptides. NPY and [Leu^{31},Pro^{34}]NPY were iodinated with Na^{125}I (Amersham) by using the oxidative agent 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Serva) as described in detail (16). The radioligands were purified by reverse-phase HPLC with a Nucleosil 300-5 C_{18} column, 0.4 × 25 cm (Macherey & Nagel), with a flow of 1 ml/min at 50°C. For NPY the column was eluted isocratically with 33.5% acetonitrile/0.1% trifluoroacetic acid/water, and for [Leu^{31}, Pro^{34}]NPY, with 34% acetonitrile. By using this procedure, it was possible to prepare different monoiodinated radioligands. The radioligands were characterized by HPLC mapping of tryptic fragments as described (17). [^{125}I-Tyr^{36}]Monoiodo-NPY and [^{125}I-Tyr^{36}]monoiodo-[Leu^{31},Pro^{34}]NPY with specific radioactivities of ≈1900 Ci/mmol (1 Ci = 37 GBq) were used as radioligands in the binding studies. In addition, [Leu^{31},Pro^{34}]NPY was iodinated to a specific activity of 18 cpm/fmol by diluting the Na^{125}I in the reaction mixture with Na^{129}I for use in saturation binding experiments.

Binding Experiments. The human, primitive neuroectodermal cell line SK-N-MC (24), the MCIX-C subclone of this, and the human neuroblastoma cell lines SMS-MSN, SMS-KAN, and CHP-234 (25) were kindly provided by June Biedler and Barbara A. Spengler (Sloan–Kettering Memorial Institute, New York). PC12 cells (26), subclone II-250, was kindly provided by Hans Thoenen (Max-Planck-Institut für Psychiatrie, Martinsried, F.R.G.). All media and materials for tissue culture were from GIBCO. As described in detail (15, 16), binding studies with cells were performed with 50,000 cpm of radioligand at 37°C, using triplicates of 1.2 × 10^6 cells that had been preincubated for 2 days in Petri dish wells, 6-well culture plates (Costar) precoated with poly(Lys-Ala) (Sigma). The incubation time was 60 min. The apparent binding at μM NPY was considered to be nonspecific binding.

Determination of $[Ca^{2+}]_i$. Confluent monolayer cultures of SK-N-MC cells were harvested 3–7 days after seeding by washing with cell medium and gently scraping with a "rubber policeman." The cells were centrifuged for 5 min at 200 × *g* and resuspended in cell medium at a concentration of 3 × 10^6 cells per ml. Fura-2 acetoxymethyl ester (Molecular Probes) was added at a final concentration of 2.5 M from a stock solution of 1 mM in dimethyl sulfoxide, and the cells were incubated for 30 min in the dark at room temperature. After centrifugation at 200 × *g* for 5 min, the cells were resuspended in Hepes buffer (145 mM NaCl/5 mM KCl/1 mM $MgSO_4$/10 mM glucose/10 mM Hepes, pH 7.4) with 150 mg of bovine serum albumin per liter at a concentration of 2.5 × 10^6 cells per ml and were used within 30 min. The Fura-2 fluorescence was measured in a Hitashi spectrofluorometer F-2000 with a monochromatic setting of 340-nm excitation and 500-nm emission; the cells were kept in suspension by gentle stirring at 37°C. The calculation of $[Ca^{2+}]_i$ was performed according to Grynkiewicz *et al.* (27) with corrections for extracellular Fura-2. The C-terminal fragment, NPY-(16–36)-peptide used in these experiments was purchased from Peninsula Laboratories.

***In Vivo* Cardiovascular Experiments.** Nonfasted female Spraque–Dawley rats, 190–210 g, were anesthetized with sodium pentobarbital, and a catheter was placed in the right jugular vein to allow the i.v. administration of test compounds. A second catheter in a carotid artery was connected to a blood pressure transducer. To facilitate respiration, the trachea was also cannulated. Blood pressure and heart rate, measured with an EKA–pulse rate meter, were continuously recorded through a dual channel recorder. After cardiovascular stabilization, each rat was treated consecutively at 10-min intervals with 0.25, 2.5, and 25 nmol of peptide per kg of body weight. When [Leu^{31},Pro^{34}]NPY was tested, an extra administration of NPY (2.5 nmol/kg) was given at the end of the experiment. The peptides were dissolved in 1 mM acetic acid and appropriately diluted in 0.05 M sodium phosphate

buffer immediately prior to the injection to obtain a dose volume of 1 ml/kg.

RESULTS

Binding of [Leu^{31},Pro^{34}]NPY to Y_1 and Y_2 Receptors. The expression of either Y_1 or Y_2 receptors on human neuroblastoma cell lines and PC-12 cells has been described (16, 17, 19). The analog [Leu^{31},Pro^{34}]NPY was found to be almost equipotent compared to authentic NPY in displacing mono[*tyrosine-36*-^{125}I]iodoNPY from cells expressing Y_1 receptors, as shown for SK-N-MC cells in Fig. 2 (*Upper*). In contrast, [Leu^{31},Pro^{34}]NPY was less potent by a factor of ≈1000 than NPY in displacing [^{125}I-Tyr^{36}]monoiodo-NPY from cells with Y_2 receptors, as shown for SMS-KAN cells in Fig. 2 *Lower*. Thus, [Leu^{31},Pro^{34}]NPY appeared to be a highly specific Y_1 receptor ligand. The displacement curves for NPY-(13–36)-peptide have been included in Fig. 2, since the binding of this long C-terminal fragment of NPY initially was used to differentiate between Y_1 and Y_2 receptor types (17, 18).

To produce a specific Y_1 receptor radioligand, [Leu^{31},Pro^{34}]NPY was radiolabeled. A complete separation of different monoiodinated species of [Leu^{31},Pro^{34}]NPY was obtained with purification by isocratic elution of the HPLC system (Fig. 3). [^{125}I-Tyr^{36}]Monoiodo-[Leu^{31},Pro^{34}]NPY was used in binding experiments. The specific binding of [^{125}I-Tyr^{36}]monoiodo-[Leu^{31},Pro^{34}]NPY to SK-N-MC cells was saturable and of high affinity as demonstrated by saturation-binding experiments performed with a specifically prepared radioligand with a low specific activity (Fig. 3 *Inset*). The dissociation constant was calculated to be 5.5 nM, and the number of binding sites was calculated to be 2.3 × 10^5 per cell. [^{125}I-Tyr^{36}]Monoiodo-[Leu^{31},Pro^{34}]NPY was displaced from the SK-N-MC cells by [Leu^{31},Pro^{34}]NPY in a similar manner as the radiolabeled standard NPY (Fig. 4 *Upper*). In contrast, [^{125}I-Tyr^{36}]monoiodo-[Leu^{31},Pro^{34}]NPY bound to SMS-KAN and CHP-234 cells in a nonspecific manner only, as it could not be displaced by [Leu^{31},Pro^{34}]NPY (Fig. 4 *Lower*).

The binding of radiolabeled as well as unlabeled [Leu^{31}, Pro^{34}]NPY was studied in several different cell lines previously described to express either Y_1 or Y_2 receptors (16, 17). As shown in Table 1, [Leu^{31},Pro^{34}]NPY behaved as a specific Y_1 receptor ligand in all cells tested. A total complementarity was found between the binding of [Leu^{31},Pro^{34}]NPY and NPY-(13–36)—i.e., cells that bound the analog with a high affinity did not bind the C-terminal fragment, and cells that bound the fragment did not bind the analog. In the present study, we did not encounter any cell line that expressed significant amounts of both receptors.

Effect of [Leu^{31},Pro^{34}]NPY on [Ca^{2+}]$_i$. Stimulation of Y_1 receptors results in the mobilization of intracellular Ca^{2+} and inhibition of forskolin-induced cAMP generation in SK-N-MC cells (U.G., L.A., J.F., T.W.S., and O.T., unpublished data). Treatment of SK-N-MC cells with [Leu^{31}, Pro^{34}]NPY was associated with a transient increase in [Ca^{2+}]$_i$ as determined in Fura-2-loaded cells (Fig. 5). A similar rise in [Ca^{2+}]$_i$ was obtained when the cells were challenged with authentic NPY. No change in [Ca^{2+}]$_i$ was observed in response to NPY-(16–36), in accordance with the fact that long C-terminal fragments do not bind to the Y_1 receptors on these cells (17, 18).

Effect of [Leu^{31},Pro^{34}]NPY on Blood Pressure. NPY is known to be a potent vasopressor agent *in vivo* (2). In anesthetized rats, both authentic NPY and [Leu^{31},Pro^{34}]NPY produced a dose-dependent increase in arterial blood pres-

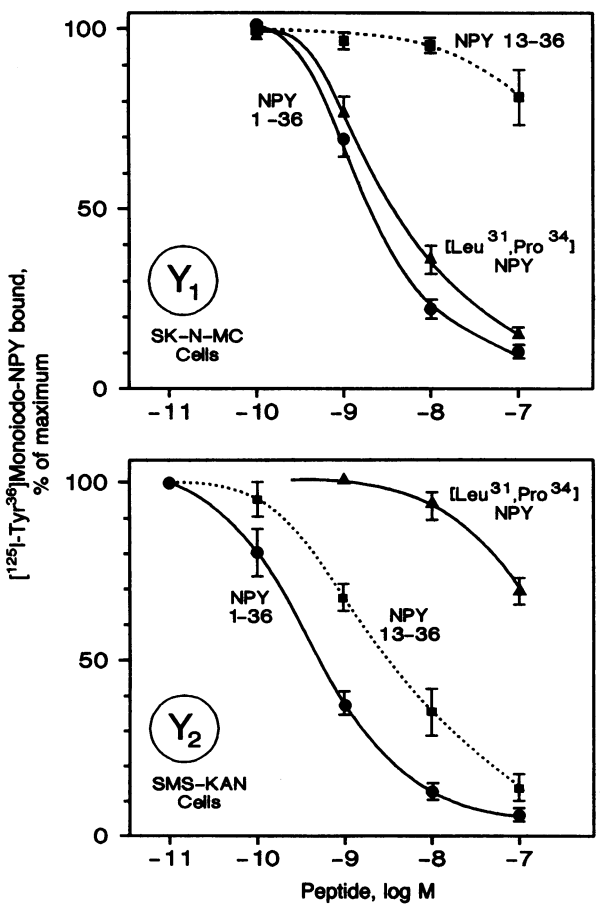

FIG. 2. Inhibition of [^{125}I-Tyr^{36}]monoiodo-NPY binding by NPY, [Leu^{31},Pro^{34}]NPY, and NPY-(13–36)-peptide. The radiolabeled NPY was displaced by NPY (●), [Leu^{31},Pro^{34}]NPY (▲), and NPY-(13–36) (■) in human neuroblastoma cell lines SK-N-MC (*Upper*) and SMS-KAN (*Lower*). Means ± SEM are shown; the number of triplicate experiments performed with each peptide in the two cell lines is indicated in Table 1. The maximal and unspecific binding of radioligand was, respectively, 14% and 3.6% to SK-N-MC cells and 8.9% and 2.6% to SMS-KAN cells.

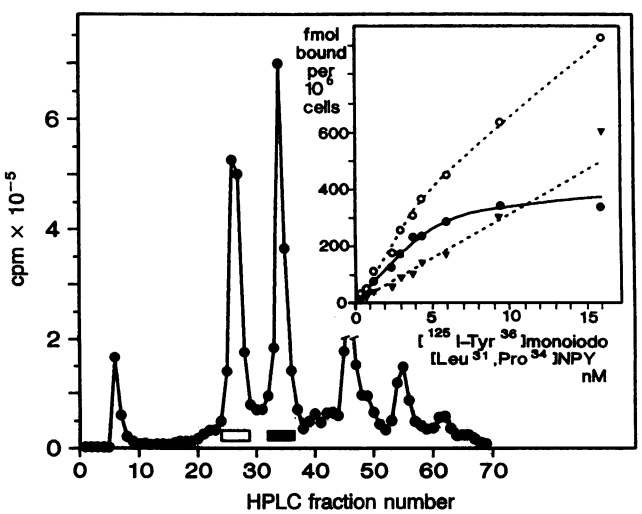

FIG. 3. Purification of [^{125}I-Tyr^{36}]monoiodo-[Leu^{31},Pro^{34}]NPY and saturation binding to SK-N-MC cells. The radioactivity profile during isocratic elution of the iodination mixture on a Nucleosil C_{18} column with 34% acetonitrile in trifluoroacidic acid 0.1% is shown. The radiolabeled peptide corresponding to the closed box was monoiodinated in position 36, and the one corresponding to the open box was monoiodinated in position one, as determined by peptide mapping. (*Inset*) Saturation binding of [^{125}I-Tyr^{36}]monoiodo-[Leu^{31}, Pro^{34}]NPY to SK-N-MC cells. The specific binding (●), total binding (○), and nonspecific binding (▼) are shown.

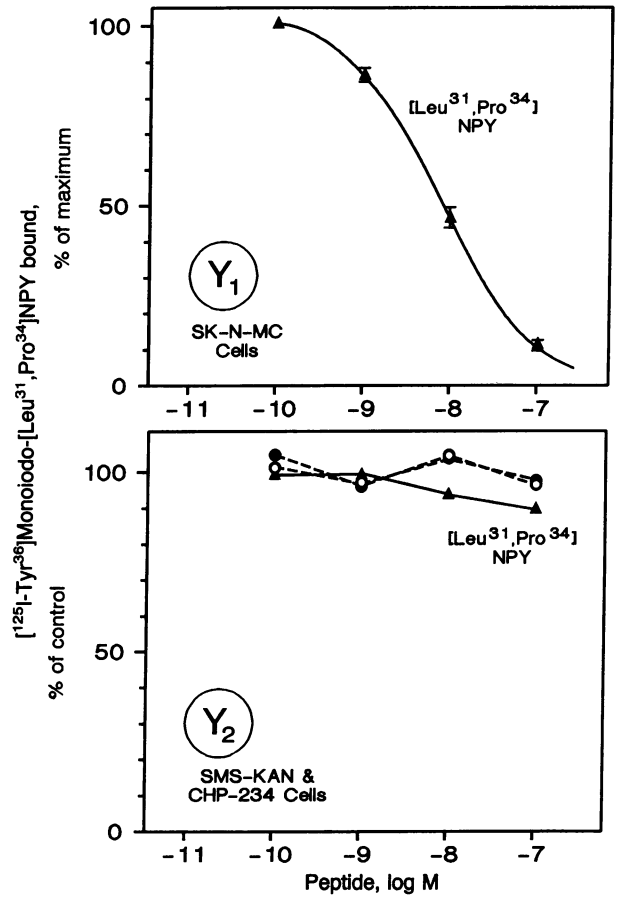

FIG. 4. Binding of [^{125}I-Tyr^{36}]monoiodo-[Leu^{31},Pro^{34}]NPY to human neuroblastoma cell lines. (*Upper*) Inhibition of [^{125}I-Tyr^{36}]monoiodo-[Leu^{31},Pro^{34}]NPY binding to SK-N-MC cells by unlabeled [Leu^{31},Pro^{34}]NPY. Means ± SEM are shown. The maximal and unspecific binding was 13.9% and 4.0%, respectively. (*Lower*) Three triplicate attempts to displace [^{125}I-Tyr^{36}]monoiodo-Leu^{31},Pro^{34}]NPY from SMS-KAN cells (▲) and from CHP-234 cells (● and ○) with the unlabeled analog. Note that the binding in *Lower* is expressed as percent of control binding with buffer only. The SMS-KAN cells bound 6.2% and CHP-234 cells bound 5.3% and 3.8% of the radioligand.

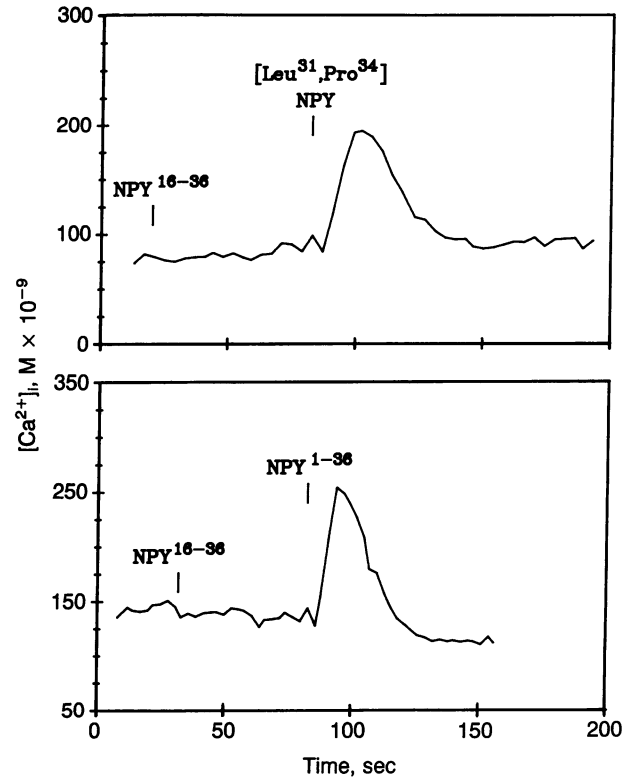

FIG. 5. Effect of [Leu^{31},Pro^{34}]NPY and NPY on $[Ca^{2+}]_i$ in SK-N-MC cells. Fura-2 was used in SK-N-MC cells in suspension (see text). The calculated $[Ca^{2+}]_i$ is plotted against time. NPY, NPY-(16–36), and [Leu^{31},Pro^{34}]NPY were added as indicated, all at 100 nM. The experiments were performed in the presence of 1 mM $CaCl_2$. The experiments shown are each representative of eight experiments.

sure (Fig. 6). In both cases, a parallel decrease in heart rate was observed, which is believed to be reflectorily related to the increase in blood pressure. At the highest dose tested, the response was more complex, as a brief increase in blood pressure was followed by a prolonged period of hypotension. It was found that [Leu^{31},Pro^{34}]NPY was ≈5 times more potent than standard NPY in causing vasoconstriction—i.e., in increasing blood pressure (Fig. 6); the difference was statistically significant at doses of 0.25 and 2.5 nmol/kg of body weight [$P < 0.05$ (Mann-Whitney)].

DISCUSSION

In the present investigation a NPY analog is described, [Leu^{31},Pro^{34}]NPY, that in both radiolabeled and unlabeled form binds exclusively to a subset of NPY receptors, called Y_1 receptors. The analog appears to be an agonist on the Y_1 receptor because it, like standard NPY, is able to mobilize intracellular Ca^{2+} in human neuroblastoma cells and because it has vasopressor activity *in vivo*.

Table 1. Binding of NPY (a protein of 36 amino acid residues), NPY-(13–36)-peptide, and [Leu^{31},Pro^{34}]NPY to cell lines

Cell lines	IC_{50} for displacing [^{125}I-Tyr^{36}]NPY, nM			IC_{50} of [Leu^{31},Pro^{34}]NPY for displacing its monoiodo derivative, nM
	NPY-(1–36)	NPY-(13–36)	[Leu^{31},Pro^{34}]NPY	
		Y_1 receptors		
SK-N-MC	2.1 (7)	>>100 (4)	3.8 (5)	9.0 (6)
MCIX-C	7.9 (14)	>>100 (5)	4.0 (3)	6.3 (1)
PC12*	4.5 (7)	>>100 (6)	7.9 (3)	ND
		Y_2 receptors		
SMS-MSN	0.16 (8)	2.0 (5)	200 (2)	ND
SMS-KAN	0.52 (3)	3.2 (5)	320 (2)	>>100 (1)
CHP-234*	0.56 (5)	11 (3)	140 (4)	>>100 (2)

The IC_{50} for the peptides in displacing radiolabeled NPY and [Leu^{31},Pro^{34}]NPY are indicated. The cell lines have been grouped according to the previous determination of their NPY receptor type (4, 16, 17). The number of triplicate experiments are indicated in parentheses. ND, not determined. *CHP-234 and PC12 cells have endogenous NPY production.

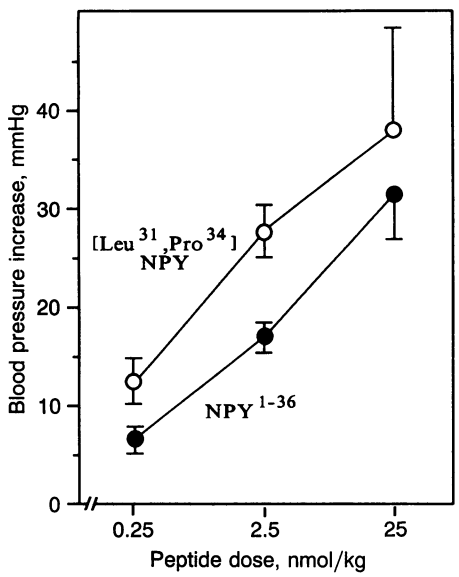

FIG. 6. Effect of [Leu^{31},Pro^{34}]NPY and NPY on blood pressure in anesthetized rats. The peptides were administered intravenously in the doses indicated at 10-min intervals. Means ± SEM are shown.

As discussed previously (4), it is likely that the Y_2 receptor is the predominating NPY receptor type in the central nervous system and that it also occurs as the major presynaptic NPY receptor in the sympathetic nervous system. Both Y_1 and Y_2 receptors appear to occur as postsynaptic receptors in the peripheral nervous system, but their relative expression seems to be both tissue- and species-dependent (4, 14, 18). Until now, the Y_1 receptor has only been defined in a rather negative manner—i.e., as an NPY receptor that did not bind long C-terminal fragments. In the present study we find a high degree of complementarity between the binding of the analog [Leu^{31},Pro^{34}]NPY and NPY-(13–36) (Fig. 2 and Table 1). Thus, [Leu^{31},Pro^{34}]NPY or variants of this could be useful in defining Y_1 receptors more specifically. Such analogs could be of importance in determining the relative importance of the receptor subtypes in the biological effects of NPY and for instance, in autoradiographical mapping of NPY receptor subtypes. For example, the finding in the present study that the Y_1-specific agonist [Leu^{31},Pro^{34}]NPY is more potent than normal NPY in increasing blood pressure may indicate that the presynatic inhibition of norepinephrine release through Y_2 receptors (18) is in fact part of the normal function of NPY.

The receptors for PP-fold peptides probably recognize the combined C-terminal and N-terminal segments of the molecules brought together by the PP-fold structure (4). This conjecture has recently been supported by two studies in which the two terminals of NPY were joined in a biologically functional way by different artificial constructions (28, 29). The C-terminal segment is important in the determination of the differential specificity as shown by the different binding of NPY and [Leu^{31},Pro^{34}]NPY to Y_2 receptors (Fig. 2 *Lower*). Of the two substitutions in the present NPY analog, we believe that the introduction of the rigid imino acid residue, proline, is the crucial structural change that is responsible for the difference in receptor binding. It is unlikely that the conservative substitution of one bulky hydrophobic residue, isoleucine, with another similar structure, leucine, could introduce such a dramatic change in binding energy. The N-terminal segment apparently is also involved in determining the differential specificity. Although PP and [Leu^{31}, Pro^{34}]NPY have the identical C-terminal sequence, they are recognized very differently both by Y_1 receptors (ref. 16, and Fig. 2 *Upper*) and by PP receptors (unpublished observations). More analogs in which also the N-terminal residues of the PP-fold peptides are exchanged are required to specifically determine the structure that delineates the different binding profiles (30) of these peptides.

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