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High-affinity neuropeptide Y receptor antagonists

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ABSTRACT **Neuropeptide Y (NPY) is one of the most abundant peptide transmitters in the mammalian brain. In the periphery it is costored and coreleased with norepinephrine from sympathetic nerve terminals. However, the physiological functions of this peptide remain unclear because of the absence of specific high-affinity receptor antagonists. Three potent NPY receptor antagonists were synthesized and tested for their biological activity in *in vitro*, *ex vivo*, and *in vivo* functional assays. We describe here the effects of these antagonists inhibiting specific radiolabeled NPY binding at Y1 and Y2 receptors and antagonizing the effects of NPY in human erythroleukmia cell intracellular calcium mobilization, perfusion pressure in the isolated rat kidney, and mean arterial blood pressure in anesthetized rats.**

Neuropeptide Y (NPY) is a 36-amino acid peptide with an N-terminal tyrosine and a C-terminal tyrosine amide, first isolated from porcine brain by Tatemoto *et al.* in 1982 (1). NPY has been found to be an abundant mammalian neuropeptide, widely distributed throughout the central and peripheral nervous systems (2–4). On the basis of the pharmacological effects observed in experimental animals after central or peripheral administration of NPY, the peptide has tentatively been implicated in the regulation of a wide variety of biological functions such as vascular tone, feeding behavior, mood, and hormone secretion among others (for a review see ref. 5). At least two NPY receptor subtypes have been described based on the relative affinity of different NPY agonists: NPY-Y1 receptors require essentially the full NPY sequence of amino acids (see Fig. 1) for activation and have high affinity for the analog [Leu^{31},Pro^{34}]NPY, whereas NPY-Y2 receptors can be activated by NPY and the shorter C-terminal fragment, NPY$_{13-36}$, but have low affinity for [Leu^{31},Pro^{34}]NPY (6, 7). A third subtype (NPY-Y3) that recognizes all three of the above peptides but is insensitive to the NPY homolog, peptide YY, has been proposed (8, 9). Direct demonstration of a physiological and pathophysiological role for NPY has been hampered by the lack of specific, high-affinity NPY receptor antagonists. Receptor antagonists based on modified C-terminal fragments of NPY (10) and analogs of the nonpeptide benextramine (11, 12) have been reported. However, these compounds have relatively weak receptor affinity and thus are of limited value. Of potential interest is the recent report by Balasubramaniam *et al.* (13) describing the antagonistic activity of [DTrp^{32}]NPY in a feeding behavior model. This manuscript reports on peptidergic high-affinity NPY receptor antagonists and their potent inhibitory action on the effects of the peptide in several *in vitro* and *in vivo* functional assays.

MATERIALS AND METHODS

Human erythroleukemia (HEL) and SK-N-MC cells were obtained from American Type Culture Collection. Cell culture medium, Eagle's minimal essential medium (EMEM) and RPMI 1640 medium, were obtained from GIBCO, bovine calf serum was from HyClone, L-glutamine solution was from JRH Biosciences (Lenexa, KS), and fura-2 acetoxymethyl ester was from Molecular Probes. All other reagents were of the highest purity available. [^{3}H]Propionyl NPY (specific activity 70 Ci/mmol; 1 Ci = 37 GBq) and ^{125}I-NPY (specific activity, 2000 Ci/mmol) were purchased from Amersham. Unlabeled porcine NPY was obtained from D. Klapper (University of North Carolina, Chapel Hill) and further purified in-house as described (14).

Peptide Synthesis. Peptides were synthesized by the solid-phase method. Compound **2** was obtained by oxidation of the reduced monomer and purification of the dimer by HPLC. Compound **3** was synthesized by using standard solid-phase synthesis. Compound **4** was synthesized by coupling Boc-L-glutamic acid fluorenylmethyl ester and α-Boc β-Fmoc-L-diamino propionic acid in position 8 and 6, respectively. Dimerization was achieved on the resin by treatment with piperidine followed by a coupling reagent. Detailed synthesis is described in the compounds' patent publication (15).

Binding Assays. [^{3}H]NPY binding to rat brain membranes was done as described (16) except that incubations were terminated by filtration on a Brandel cell harvester through a Whatman GF/B filter, previously soaked overnight in 0.3% polyethylenimine. Samples were washed three times with 4 ml of wash buffer [20 mM Hepes buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 0.05% bovine serum albumin (BSA)] and each filter disc was then placed in a scintillation vial, soaked overnight in 5 ml of Scintiverse (Fisher), and assayed in a Beckman LS5801 liquid scintillation counter. Specific binding was defined as that displaced by excess (100 nM) unlabeled porcine NPY and represented 65% of total binding at a ligand concentration of 0.2 nM. All conditions were tested in triplicate. Displacement curves were obtained by incubation of various dilutions of the test compounds in the presence of 0.2 nM [^{3}H]NPY and the IC_{50} values were calculated from nonlinear regression analysis (Hill plot) of the data.

Binding of ^{125}I-NPY to NPY-Y1 receptors in intact human neuroblastoma SK-N-MC cells was done following the procedure of Gordon *et al.* (17) with minor modifications. Cells were grown and maintained in EMEM supplemented with 25 mM Hepes, 2 mM glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, and 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. Cells were grown to confluency in multiwell plates (24 × 16 mm polystyrene), washed twice with EMEM, and then equilibrated for 30 min at room temperature in EMEM containing 25 mM Hepes, 0.5% BSA, 50 μM phenylmethylsulfonyl fluoride, and 0.1% bacitracin (binding buffer). Then cells were incubated for 3 hr in 0.5 ml of binding buffer containing ^{125}I-NPY at concentrations ranging from 0.05 to 10 nM. Specific binding was defined as that

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Abbreviations: NPY, neuropeptide Y; HEL, human erythroleukemia; MAP, mean arterial pressure.

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displaced by excess (1 μM) unlabeled porcine NPY and represented 87% of the total binding at a ligand concentration of 0.7 nM. Incubations were terminated by aspiration of the medium followed by three washes with ice-cold phosphate-buffered saline. Cells were solubilized in 0.1 M NaOH and an aliquot was taken for determination of radioactivity in an LKB γ counter. All conditions were tested in triplicate. Displacement curves were obtained by incubation of various dilutions of the test compounds in the presence of 0.7 nM ^{125}I-NPY under the same experimental conditions as above. The IC_{50} values for NPY and test compounds, for the displacement of specifically bound ^{125}I-NPY, were calculated from nonlinear regression analysis (Hill plot) of the data.

Receptor binding assays for profiling were done following previously published standard procedures.

Intracellular Calcium Measurements. HEL cells were loaded with fura-2 acetoxymethyl ester (1 μM), washed by centrifugation, and suspended in buffer at 10^6 cells per ml as described (18). Fluorescence was measured at room temperature in an SLM Aminco DMX-1000 spectrofluorometer, with dual excitation at 340 and 380 nm and emission recording at 510 nm, using 0.5 ml of cell suspension and buffer up to a total volume of 2.5 ml, with constant stirring. Compounds (0.05 nM–1 μM, final concentration) were added to the medium 30 sec prior to stimulation with a half-maximal concentration (5 nM) of NPY and results were recorded as the ratio of fluorescence at 340/380 and converted into cytosolic calcium concentrations with the aid of an SLM Aminco proprietary software as detailed elsewhere (18).

Isolated Rat Kidney Perfusion. Male Sprague–Dawley rats (200–400 g) were anesthetized by i.p. administration of a mixture of acepromazine (2.8 mg/kg) and ketamine (112 mg/kg). The abdomen was opened and the renal arteries and descending aorta were cleaned. With minimal disruption of blood flow, the left kidney was cannulated through the proximal renal artery using a stainless steel 20-gauge blunt-tipped needle attached to tygon perfusion tubing and to a peristaltic pump. The perfusion of the kidney was started (10 ml/min) with a solution containing 120 mM NaCl, 5.0 mM KCl, 15.5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.5 mM glucose, and 1.0% BSA. The perfusion solution was saturated with 95% O_2, adjusted to pH 7.4, and maintained at 37°C. NPY and other pressor substances were injected as a bolus (0.1 ml) through an injection loop placed a short distance from the kidney. Compounds were perfused into the kidney for 5 min before and during the stimulation with NPY to allow enough time to obtain a stable concentration of drug in the perfusion fluid and tissue equilibration. Immediately after stimulation, the NPY receptor becomes desensitized but the NPY response fully recovers within 30 min. Stimulation can be repeated with consistent responses every 30 min for 3–4 hr.

Mean Arterial Blood Pressure in Anesthetized Rats. Sprague–Dawley rats (350–450 g) were anesthetized with Inactin (thiobutabarbital sodium; 100 mg/kg), tracheostomized, and ventilated using a respiratory pump. Body temperature was maintained at 37°C with a heating pad. The left femoral artery was cannulated and arterial blood pressure was monitored using a Statham P23 ID pressure transducer and recorded on a Grass polygraph (model 7D). The femoral vein was cannulated to allow for the administration of drugs. Porcine NPY was administered as an i.v. bolus (1 nmol/kg) and peak changes in mean arterial pressure (MAP) were recorded. 1229U91 (compound **4**) was infused i.v. at half-logarithmic incremental doses (0.1–10 nmol/kg per min). Each dose was infused over a period of 10 min at a rate of 0.1 ml/min, providing a total dose of 1–100 nmol/kg at the end of the infusion period. A test dose of NPY was administered 1 min after the end of the compound infusion. All peptides were dissolved in isotonic saline containing 0.05% BSA. All experiments involving animals were performed following the guidelines established by the internal animal care and use committee at Burroughs Wellcome.

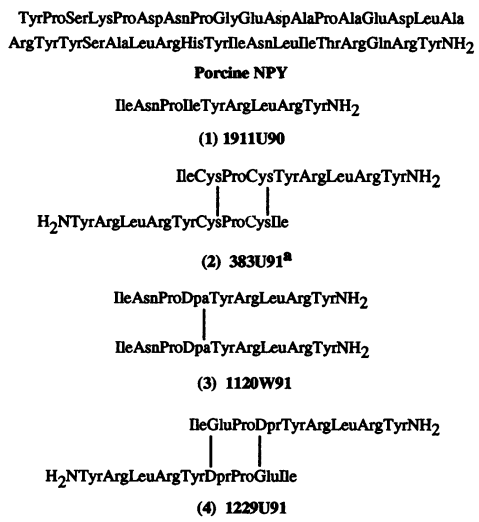

FIG. 1. Peptide sequence for porcine NPY and antagonists. Dpa, (+)-2,6-diaminopimelic acid; Dpr, 2,3-diaminopropionic acid. a, The compound was obtained as an equal mixture of the parallel and antiparallel isomers.

RESULTS

We have recently reported (16) on the structure and activity of a nonapeptide (see compound **1**, Fig. 1) displaying high affinity for rat brain receptors (IC_{50} = 180 nM) and potent antagonistic activity (IC_{50} = 10 nM) in the NPY-Y1 receptor-mediated intracellular calcium increase in HEL cells. In an attempt to improve on the potency of the above antagonist, we synthesized dimeric analogs of **1** using either disulfide bridges, diamino pimelic acid, or lactam bridges (Fig. 1, compounds **2**, **3**, and **4**, respectively). Table 1 shows the activity of these compounds, compared to that of NPY and NPY analogs, displacing specifically bound [^{3}H]NPY from rat brain membranes or ^{125}I-NPY from SK-N-MC neuroblastoma cells. The three dimeric structures show significantly higher affinities than compound **1** for Y2 (rat brain) and Y1 (SK-N-MC cells) receptors; thus, these dimers are potent, albeit apparently nonselective, NPY receptor ligands. Even though our compounds do not appear to be selective for a particular NPY receptor subtype, their apparent affinity for many other neurotransmitter or peptide receptors is several orders of magnitude weaker than for NPY receptors (see Table 2).

Next, we examined the ability of these compounds to inhibit the NPY-induced increase in cytosolic calcium in HEL cells. We have previously shown that HEL cells mobilize intracel-

Table 1. Displacement of specifically bound [^{3}H]NPY from rat brain membranes and ^{125}I-NPY from SK-N-MC cells by NPY, NPY analogs, and compounds **2**, **3**, and **4**

	IC_{50}, nM	
	Rat brain	SK-N-MC
NPY	0.45 ± 0.06 (19)	1.33 ± 0.3 (6)
[Leu^{31},Pro^{34}]NPY	2.6 ± 0.6 (8)	0.62 ± 0.05 (3)
NPY_{13-36}	15 ± 2.0 (5)	623 ± 57 (4)
Compound **2**	8.8 ± 1.7 (10)	2.9 ± 0.5 (4)
Compound **3**	1.3 ± 0.29 (8)	0.73 ± 0.18 (5)
Compound **4**	0.021 ± 0.006 (16)	0.20 ± 0.01 (3)

Results are expressed as the IC_{50} calculated from nonlinear regression analysis of the concentration-dependent displacement data. Results are expressed as the mean ± SE of experiments (*n*) in triplicate.

Table 2. Receptor binding profile for compounds **2**, **3**, and **4**

Receptor	Tissue, ligand	% inhibition Compound **2**	Compound **3**	Compound **4**
α_1	Rat brain, [^{3}H]WB4101 (0.2 nM)	(6.9 μM)	(10 μM)	(4.6 μM)
α_2	Rat brain, [^{3}H]clonidine (4 nM)	21	NT	13.5
β	Rat cerebral cortex, [^{3}H]dihydroalprenolol (5 nM)	2	NT	19
Benzodiazepine	Rat forebrain, [^{3}H]diazepam (1.5 nM)	0	0	2
PAF	Rabbit platelets, [^{3}H]PAF (0.2 nM)	0	0	0
Angiotensin II	Rat liver, [^{3}H]angiotensin II (1 nM)	0	15	2
Dopamine (D2)	Rat striatum, [^{3}H]spiroperidol (1 nM)	8	34	11
5-HT$_{1A}$	Rat hippocampus, [^{3}H]-8-OH-DPAT (0.2 nM)	8.5	33	7
5-HT$_2$	Rat frontal cortex, [^{3}H]ketanserin (0.5 nM)	21	53	58
Adenosine 1	Rat brain, [^{3}H]cyclohexyladenosine (3 nM)	20	NT	11
Adenosine 2	Rat striatum, [^{3}H]ethylcarboxamide adenosine (4 nM)	16	NT	11
Neurotensin	Rat forebrain, [^{3}H]neurotensin (2 nM)	17	NT	(7.3 μM)
Ca^{2+} channel (dihydropyridine)	Rat cerebral cortex, [^{3}H]nitrendipine (0.2 nM)	27	NT	3
Ca^{2+} channel (phenylalkylamine)	Rat heart, [^{3}H]verapamil (1 nM)	10	NT	1
Glutamate	Rat forebrain, [^{3}H]glutamate (5 nM)	12	NT	38
Cholinergic M1	Rat forebrain, [^{3}H]pirenzepine (1 nM)	(2.6 μM)	NT	NT
Cholinergic M2	Rat heart, [^{3}H]QNB (0.05 nM)	(2.2 μM)	NT	NT

Individual assays were performed using previously described standard procedures. Data are presented as the percent inhibition of the specific binding of the radioactive ligand in the presence of the compounds (10 μM) or, in parentheses, as the concentration that inhibits specific binding by 50% (IC_{50}). 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxydipropylaminotetralin; WB4101, [(2,6-dimethoxyphenoxyethyl)amino methyl-1,4-benzadioxane]; PAF, platelet-activating factor; QNB, quinuclidinyl benzilate; NT, not tested.

lular calcium from an inositol 1,4,5-trisphosphate-sensitive pool in response to the activation of a NPY-Y1 type of receptor coupled to phospholipase C (14, 18). Fig. 2 *Left* shows the effect of a 0.5 nM dose of each compound, added to HEL cells in suspension, 30 sec prior to the stimulation with a half-maximal concentration of NPY (5 nM). It can be observed that, at this concentration, the three compounds inhibit very effectively (40–80%) the increase in cytosolic calcium induced by NPY. Fig. 2 *Inset* shows the IC_{50} for the compounds calculated from the dose–response curves for the inhibition of the calcium response to 5 nM NPY for each of the antagonists. None of the compounds has agonistic activity, as no increase in cytosolic calcium is observed in response to their addition to the cell suspension prior to NPY (even up to 1 μM), indicating that these compounds would behave as pure antagonists at the NPY receptor expressed in HEL cells. The antagonistic potency of the three compounds is very similar although slightly higher for compound **4**. Inhibition by compound **4** appears to be competitive in nature as suggested from the NPY dose–response curve in the presence and absence of the inhibitor (Fig. 2 *Right*).

The vasculature of the isolated rat kidney contracts intensively in response to a bolus injection of NPY or [Leu^{31}, Pro^{34}]NPY (Y1 specific) but not to NPY$_{13-36}$ (Y2 specific), producing an immediate increase in perfusion pressure. Fig. 3, *Left* shows the time course of the pressor effect of a single bolus injection of a half-maximal dose (50 pmol) of NPY. Stimulation with a 50 pmol bolus of NPY while the preparation is being infused with the aforementioned antagonists at a concentration of 5 nM (*Right*) results in a marked inhibition of the pressor effect of NPY. The three antagonists potently inhibit the NPY-induced vasoconstriction; however, compound **4**

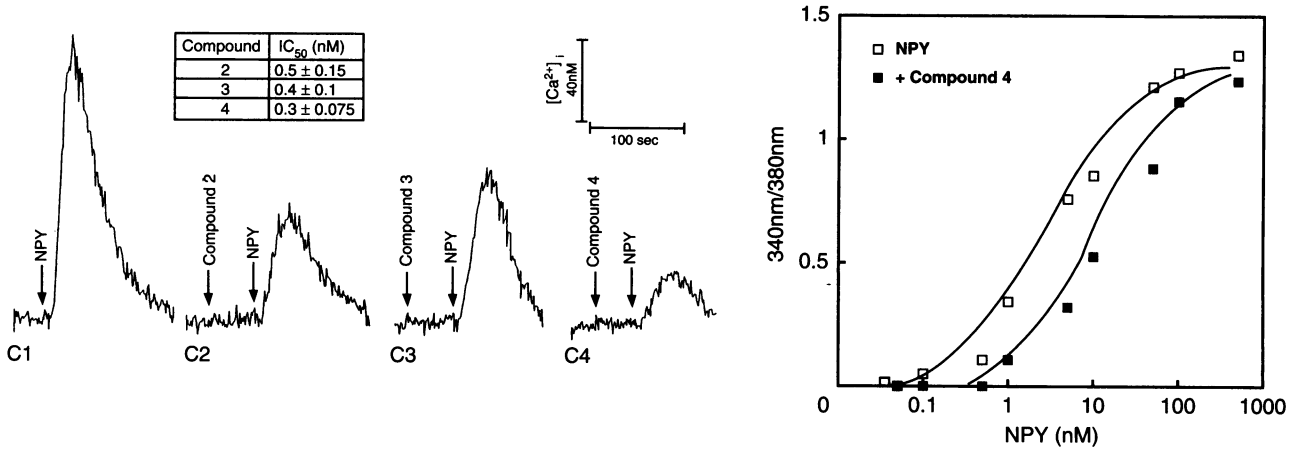

FIG. 2. Inhibition of the NPY-induced increase in cytosolic calcium in fura-2-loaded HEL cells by compounds **2**, **3**, and **4**. C1, C2, C3, and C4 represent four different cuvettes. (*Left*) C1, cytosolic calcium response to a half-maximal concentration of NPY (5 nM); C2–C4, cytosolic calcium response to 5 nM NPY 30 sec after the addition of compounds **2**, **3**, and **4**, respectively. The final concentration of antagonist was 0.5 nM. (*Inset*) IC_{50} for each compound, calculated from the dose–response curves (n = 3), for the inhibition of the calcium response to 5 nM NPY. (*Right*) NPY dose–response curves in the presence and absence of compound **4** at 0.25 nM.

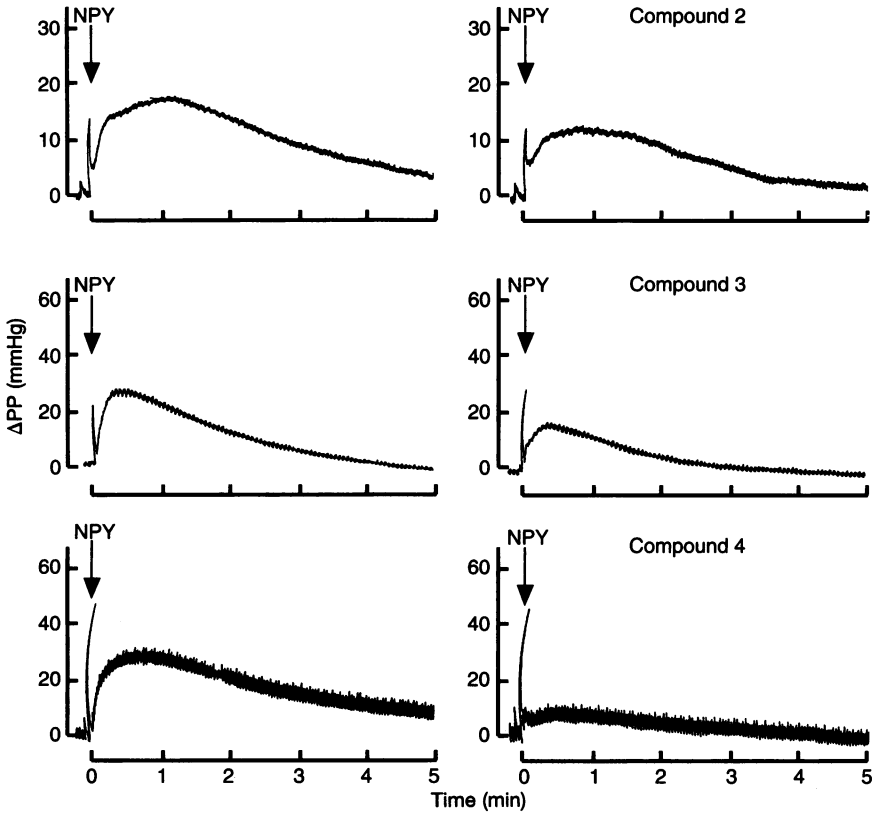

FIG. 3. Inhibition of the NPY-induced increase in perfusion pressure (PP) in the isolated rat kidney. The kidneys were perfused at constant flow rate for 30 min before the first challenge with a bolus injection of 50 pmol of NPY in 0.1 ml of perfusion medium (*Left*). Following a 30-min recovery period, compounds **2**, **3**, and **4** were infused for 5 min at a concentration of 5 nM. After 4 min of infusion with the antagonists, the kidney was challenged again with 50 pmol of NPY (*Right*). The infusion was discontinued 1 min after the stimulation with NPY. Each of the compounds was tested in a different kidney preparation. Results represent typical experiments repeated at least twice with identical results. IC_{50} values for these compounds were calculated from dose–response curves ($n = 3$) for the inhibition of the increase in perfusion pressure in response to 50 pmol of NPY for each of the antagonists (not shown) and were 5.5 ± 2.5 nM, 4.5 ± 1.9 nM, and 2.2 ± 0.6 nM, for compounds **2**, **3**, and **4**, respectively.

shows a higher antagonistic potency in this system as well. Supporting the specificity of our compounds for NPY receptors, increases in kidney perfusion pressure induced by methoxamine (α_1 agonist), isoproterenol (β agonist), or depolarization with KCl are not affected by the presence of these antagonists (results not shown).

NPY has been shown to be a potent vasoconstrictor *in vivo*, elevating arterial blood pressure in rats (reviewed in ref. 19). Fig. 4 shows the *in vivo* NPY receptor blocking effect of compound **4**, which exhibits the greatest potency in all of the above assays. i.v. infusion of compound **4** into anesthetized Sprague–Dawley rats dose dependently inhibits the pressor response produced by a bolus i.v. injection of an ED_{50} dose of NPY (1 nmol/kg). This antagonism is selective for the NPY receptors since the pressor response induced by a bolus i.v. injection of norepinephrine (3 nmol/kg) is not altered.

DISCUSSION

Initial hypotheses regarding the physiological role(s) for NPY have been based on the *in vivo* pharmacological effects observed after exogenous administration of the peptide. Wahlestedt *et al.* (20) have recently provided convincing evidence that NPY may be involved in the mechanism of anxiety, circumventing the lack of specific NPY receptor antagonists by using an antisense oligonucleotide to the rat Y1 receptor. In addition, intrahypothalamic administration of monoclonal antibodies to NPY produces a marked reduction in food intake in the fasted rat (21, 22), suggesting a physiological role for NPY in the control of feeding behavior after a period of food deprivation. However, in order to ascribe a true physiological function to a neuropeptide we must ultimately rely on the changes observed after neutralizing the effect of the endogenously released peptide with potent and specific receptor antagonists.

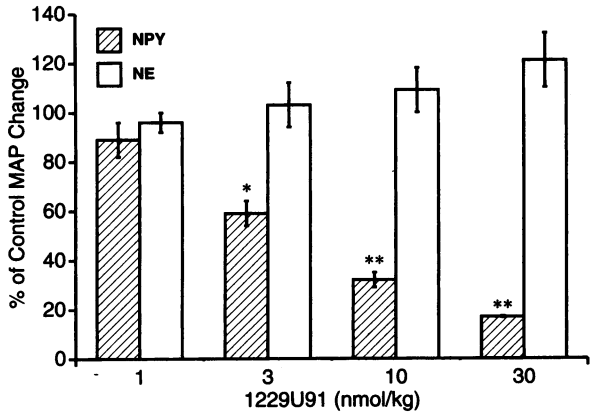

FIG. 4. Effect of compound **4** on the pressor responses induced by NPY and norepinephrine (NE) in inactin-anesthetized rats. 1229U91 was administered as an i.v. infusion at half-logarithmic incremental doses (0.1–10 nmol/kg per min) over a 10-min period. Porcine NPY (1 nmol/kg) and NE (3 nmol/kg) were administered as an i.v. bolus, 1 min after the end of the compound infusion, and peak changes in MAP were recorded. Note the absence of any effect on the pressor response to i.v. NE. Control MAP changes to NPY and NE were 28 ± 1 and 56 ± 8 mmHg, respectively. Results are shown as percent change of control (mean ± SE; $n = 5$). *, $P < 0.05$; **, $P < 0.01$.

The three dimeric structures reported here show high affinities for both Y1 and Y2 type of NPY receptors. Scatchard analysis (not shown) of the rat brain membrane binding data indicates, under our experimental conditions, an apparent homogeneous population of receptors with a $K_d = 0.36 \pm 0.03$ nM and a $B_{max} = 306 \pm 17$ fmol/mg of protein (mean ± SE; $n = 15$). A similar analysis on the SK-N-MC cell data shows a single binding site with a $K_d = 0.7 \pm 0.072$ nM and a $B_{max} = 39.1 \pm 7.9$ fmol per 10^6 cells (mean ± SE; $n = 5$). Moreover, the Hill slope for the NPY displacement curves in both systems is consistent with a single binding site (nH = 0.99 ± 0.01 and 0.89 ± 0.17 for brain membranes and SK-N-MC cells, respectively). The differential affinity of the C-terminal fragment NPY_{13-36} and the analog [Leu^{31},Pro^{34}]NPY would indicate that the Y2 type receptor predominates in the rat brain preparation while SK-N-MC cells would express the Y1 type; thus, our antagonists do not appear to be selective, at least between these two receptors. It is worth noting that compound **4** shows a higher apparent affinity for NPY receptors (particularly from rat brain) than NPY itself, suggesting that a dimeric structure may induce a preferred conformation for high-affinity binding to NPY receptors. It has been suggested that helicity of the C-terminal end of NPY may play an important role in receptor recognition (reviewed in ref. 23). Preliminary information on the solution structure of compound **4** indicates the presence of an extended helical structure (unpublished data).

The potency of the dimers on the inhibition of the NPY-induced increase in cytosolic calcium correlates well with their potency antagonizing the pressor effect of NPY in anesthetized rats; thus, HEL cells may constitute a good model to screen for NPY antagonists as potential antihypertensive agents. We have shown here that compound **4** is very effective in inhibiting the vasoconstrictor effect of NPY in the isolated perfused kidney as well as the NPY-induced increase in peripheral resistance and increase in blood pressure in rats. It is noteworthy, however, that resting MAP is not significantly affected by compound **4** (−9 ± 7% at the highest dose), suggesting that endogenous NPY is not a major contributor to the cardiovascular tone in anesthetized rats.

The compounds described here, particularly 1229U91 (compound **4**), represent a first generation of high-affinity NPY antagonists that will help unveil the participation of NPY in important physiological functions and in the development of therapeutic agents to treat central as well as peripheral pathologies associated with an NPYergic transmission hyperactivity. While this manuscript was in preparation, Rudolf *et al.* (24) reported on a nonpeptide NPY antagonist apparently selective for the NPY-Y1 type of receptor.

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