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GR231118 (1229U91) and other analogues of the C-terminus of neuropeptide Y are potent neuropeptide Y Y_1 receptor antagonists and neuropeptide Y Y_4 receptor agonists

Eric M. Parker [a,*], Carol K. Babij [a], Ambikaipakan Balasubramaniam [b], Robert E. Burrier [a,1], Mario Guzzi [a], Fozia Hamud [a], Gitali Mukhopadhyay [a], Mark S. Rudinski [a], Z. Tao [b], Melissa Tice [a], Ling Xia [a], Deborra E. Mullins [a], Brian G. Salisbury [a]

[a] *Department of Central Nervous System and Cardiovascular Research, Schering-Plough Research Institute, Mail Stop K-15-3-3600, 2015 Galloping Hill Road, Kenilworth, NJ 07033-0539, USA*

[b] *Division of Gastrointestinal Hormones, Department of Surgery, University of Cincinnati Medical Center, Cincinnati, OH 45267, USA*

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Abstract

GR231118, BW1911U90, Bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y(31–36)} (T-190) and [Trp–Arg–Nva–Arg–Tyr]$_2$-NH_2 (T-241) are peptide analogs of the C-terminus of neuropeptide Y that have recently been shown to be antagonists of the neuropeptide Y Y_1 receptor. In this study, the activity of these peptides at each of the cloned neuropeptide Y receptor subtypes is determined in radioligand binding assays and in functional assays (inhibition of forskolin-stimulated cAMP formation). GR231118 is a potent antagonist at the human and rat neuropeptide Y Y_1 receptors ($pA_2 = 10.5$ and 10.0, respectively; $pK_i = 10.2$ and 10.4, respectively), a potent agonist at the human neuropeptide Y Y_4 receptor ($pEC_{50} = 8.6$; $pK_i = 9.6$) and a weak agonist at the human and rat neuropeptide Y Y_2 and Y_5 receptors. GR231118 also has high affinity for the mouse neuropeptide Y Y_6 receptor ($pK_i = 8.8$). Therefore, GR231118 is a relatively selective neuropeptide Y Y_1 receptor antagonist, but has appreciable activity at the neuropeptide Y Y_4 and Y_6 receptors as well. BW1911U90, T-190 and T-241 are moderately potent neuropeptide Y Y_1 receptor antagonists ($pA_2 = 7.1$, 5.8 and 6.5, respectively; $pK_i = 8.3$, 6.5 and 6.8, respectively) and neuropeptide Y Y_4 receptor agonists ($pEC_{50} = 6.8$, 6.3 and 6.6, respectively; pK_i;8.3, 7.7 and 8.3, respectively). These data suggest that the C-terminus of neuropeptide Y and related peptides is sufficient for activation of the neuropeptide Y Y_4 receptor, but is not sufficient for activation of the neuropeptide Y Y_1 receptor. Because BW1911U90, T-190 and T-241 are significantly less potent at the cloned human neuropeptide Y Y_1 receptor than at the neuropeptide Y receptor in human erythroleukemia cells, these cells may express a novel neuropeptide Y receptor with high affinity for these peptides.

Keywords: Neuropeptide Y; GW1229; 1229U91; GR 231118; Neuropeptide Y receptor

1. Introduction

Neuropeptide Y is a 36 amino acid neuropeptide that is widely distributed in the central and peripheral nervous systems (Wahlestedt and Reis, 1993). As might be expected from its wide distribution, neuropeptide Y has a plethora of physiological effects. In the central nervous system, neuropeptide Y stimulates food intake, decreases thermogenesis, modulates pituitary hormone secretion and has anxiolytic and anti-convulsant activity (Turton et al., 1997; Woldbye et al., 1997). In the periphery, neuropeptide Y has been shown to regulate vascular tone and to contract or relax smooth muscle in a variety of tissues (Wahlestedt et al., 1990; Malmstrom and Lundberg, 1997). The multiple physiological effects of neuropeptide Y are mediated by at least six different G protein-coupled receptors designated Y_1, Y_2, Y_3, Y_4, Y_5 and Y_6 (Michel et al., 1998). With the exception of the neuropeptide Y Y_3 receptor, each of these neuropeptide Y receptors has been cloned. There may also be additional neuropeptide Y receptors that have not yet been identified by molecular cloning (Michel et al., 1998). Unequivocal determination of the neuropeptide Y receptors that mediate the various

* Corresponding author. Tel.: +1-908-298-7389; fax: +1-908-298-2383; e-mail: eric.parker@spcorp.com

[1] Present Address: Lead Generation Biology, Sphinx Pharmaceuticals, 4615 University Drive, Durham, NC 27707, USA.

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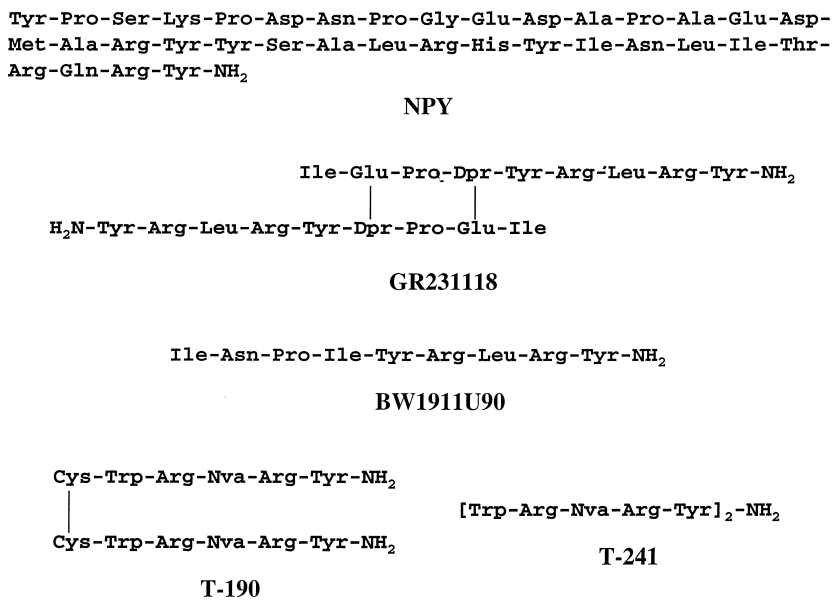

Fig. 1. Structures of GR231118, BW1911U90, T-190 and T-241.

physiological effects of neuropeptide Y would be greatly facilitated by the availability of selective agonists and antagonists for each neuropeptide Y receptor.

Recently, peptides such as GR231118 (also known as 1229U91 and GW1229), BW1911U90, Bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}] neuropeptide Y(31–36)}(T-190) and [Trp–Arg–Nva–Arg–Tyr]$_2$-NH_2 (T-241) (Fig. 1) have been reported to be potent and selective neuropeptide Y Y_1 receptor antagonists (Leban et al., 1995; Daniels et al., 1995; Balasubramaniam et al., 1996a,b). These peptides share the common feature of being analogues of the C-terminus of neuropeptide Y. The ability of GR231118 to antagonize various physiological actions of neuropeptide Y has been used to ascribe these actions of neuropeptide Y to activation of the neuropeptide Y Y_1 receptor (e.g., Kanatani et al., 1996; Hegde et al., 1995; Lew et al., 1996). Hegde et al. (1995) have shown that GR231118 does not have significant affinity for the neuropeptide Y Y_2 receptor in SMS-KAN cells, but the activity of GR231118 at the recently cloned neuropeptide Y Y_4 and Y_5 receptors has not been reported. Thus, the selectivity of GR231118 for the neuropeptide Y Y_1 receptor remains to be conclusively demonstrated. This study presents a complete pharmacological characterization of GR231118, BW1911U90, T-190 and T-241 at each of the cloned neuropeptide Y receptor subtypes.

2. Materials and methods

2.1. Materials

GR231118 (also known as 1229U91 and GW1229) and BW1911U90 were synthesized by Anaspec (San Jose, CA). T-190 and T-241 were synthesized by step-wise solid phase methodology using an automated Applied Biosystem Model 430A instrument. Briefly, *tert*-butyloxy-carbonyl amino acids with benzyl- or halobenzyl-based side chain protecting groups were assembled sequentially on para-methylbenzhydrylamine resin according to the standard program provided by the manufacturer for the generation and coupling of preformed 1-hydroxy-benzotriazole esters. At the end of the synthesis, the *N*-indole formyl group was removed by stirring the resin with 20% piperidine in dimethylformamide for 2 h, and the free peptide was obtained by hydrofluoric acid cleavage as described earlier (Balasubramaniam et al., 1996a). The crude peptide was purified by semi-preparative reversed phase chromatography using previously published procedures (Balasubramaniam et al., 1996a). The purified peptide was characterized by amino acid and mass spectral analyses. All other peptides used in this study were purchased from Bachem (King of Prussia, PA). Chinese hamster ovary (CHO-K1), human embryonic kidney 293, COS1, SK-N-MC and SMS-KAN cells were obtained from American Type Culture Collection (Rockville, MD).

2.2. Cloning of the neuropeptide Y Y_1, Y_2, Y_4 and Y_6 receptors

Total RNA was isolated from the SK-N-MC cells, SMS-KAN cells and rat hypothalamus with the Tri Reagent kit (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Poly A^+ RNA was isolated from total RNA with the Fast Track 2.0 kit (InVitrogen, San Diego, CA) according to the manufacturer's instructions. Rat lung poly A^+ RNA, rat genomic

DNA, mouse genomic DNA and human genomic DNA were obtained from Clontech (Palo Alto, CA). cDNA was prepared by reverse transcription of poly A^+ RNA with the GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT) according to the manufacturer's instructions. The following oligonucleotide primers were used to prime reverse transcription: human neuropeptide Y Y_1 receptor, GGGGATCCATGAATTCAACATTATTTTCCCAGGTTGAAAATCATTCAG; rat neuropeptide Y Y_1 receptor, TTCCAAACCTGCCTCAATCAA; human neuropeptide Y Y_2 receptor, GGGTGTAGAGAGCCGCCATCAACCAGATTC; rat neuropeptide Y Y_4 receptor, AGGGTGTGTCGAAAGAAAAT. Neuropeptide Y receptors were cloned by the polymerase chain reaction (PCR) from the following cDNA or genomic DNA preparations: human neuropeptide Y Y_1 receptor, SK-N-MC cell cDNA; rat neuropeptide Y Y_1 receptor, rat hypothalamus cDNA; human neuropeptide Y Y_2 receptor, SMS-KAN cell cDNA; rat neuropeptide Y Y_2 receptor, rat genomic DNA; human neuropeptide Y Y_4 receptor, human genomic DNA; rat neuropeptide Y Y_4 receptor, rat lung cDNA; mouse neuropeptide Y_6 receptor, mouse genomic DNA. The following primer pairs were used in the PCR: human neuropeptide Y Y_1 receptor, GGCAAGCTTCAGATTTTTTCATTATCATCATTGTTG and GGGGATCCATGAATTCAACATTATTTTCCCAGGTTGAAAATCATTCAG; rat neuropeptide Y Y_1 receptor, CTAATCCATAACAACAAAACG and TTCCAAACCTGCCTCAATCAA; human neuropeptide Y Y_2 receptor, GGGGTCGACTGAAAATGGGTCCAATAGGTG and GGGTGTAGAGAGCCGCCATCAACCAGATTC; rat neuropeptide Y Y_2 receptor, GGGATCCGTTGTTAACAGACTCGTGTAAAG and GGGGTCTAGATGATGCTTTTAGGGTCAATACAAT; human neuropeptide Y Y_4 receptor, GGGGTCGACATGAACACCTCTCACCTGGCCTTG and GGGGCGGCCGCTTAAATGGGATTGGACCTGCCACTTA; rat neuropeptide Y Y_4 receptor, CACCCACCATGAATACCTCT and AGGGTGTGTCGAAAGAAAAT; mouse neuropeptide Y Y_6 receptor, GGAAGCTTAGAAAATCTCTAATTAAAATCC and GCTTTGATTTTGCTTACC. The resulting PCR products were then directly cloned into the expression vectors pcDNA3.1 (InVitrogen), pCR3.1 (InVitrogen) or pCIneo (Promega, Madison, WI). DNA sequencing demonstrated that each of the neuropeptide Y receptor sequences was identical to the published sequences (Eva et al., 1990; Larhammar et al., 1992; Rose et al., 1995; Gerald et al., 1995; Yan et al., 1996; Weinberg et al., 1996).

2.3. Cloning of the rat neuropeptide Y Y_5 receptor

Rat brain poly A^+ RNA was purchased from Clontech. Rat brain cDNA was prepared by reverse transcription with the GeneAmp RNA PCR Kit according to the manufacturer's instructions. Reverse transcription was primed with an oligonucleotide with the sequence GGGCTCGAGGCACAGAGAGAATCATGACATGTG. The rat neuropeptide Y Y_5 receptor cDNA was isolated from rat brain cDNA via the PCR. The oligonucleotide primers used in the PCR were CGCGGATCCCCGAGGTGCTTCTAAAAC and GGGCTCGAGGCACAGAGAGAATCATGACATGTG. The resulting PCR product was subcloned into pcDNA3.1. The sequence of the rat neuropeptide Y Y_5 receptor cDNA isolated in the PCR was identical to nucleotides 71–1591 of the sequence reported by Hu et al. (1996) with two exceptions. First, the nucleotide at position 228 of the sequence of Hu et al. (1996) (position-5 relative to the ATG initiation codon) was found to be a C rather than a T as reported by Hu et al. (1996). This substitution was seen in multiple clones isolated from independent PCR reactions. Second, the rat neuropeptide Y Y_5 receptor cDNA isolated by the PCR had a 123 base pair insertion between nucleotides 232 and 233 (positions-9 and -10 relative to the ATG initiation codon) of the sequence of Hu et al. (1996). This insertion was also present in some of the rat neuropeptide Y Y_5 receptor cDNA clones isolated by Hu et al. (1996), although its sequence has not been published. The sequence of the rat neuropeptide Y Y_5 receptor cDNA used in this study has been deposited in the GenBank database (accession number AF044264). The rat neuropeptide Y Y_5 receptor cDNA was then modified by removing the 5′ untranslated sequence and adding a Kozak consensus sequence for translation initiation (Kozak, 1987). This was accomplished by the PCR. The oligonucleotide primers used in the PCR reaction were TTTGGATCCACCATGGAGTTTAAG and GGGCTCGAGGCACAGAGAGAATCATGACATGTG. The resulting PCR product was subcloned into the expression vector pCIneo.

2.4. Cloning of the human neuropeptide Y Y_5 receptor and construction of a modified human neuropeptide Y Y_5 receptor

SK-N-MC cell cDNA was prepared by reverse transcription with the GeneAmp RNA PCR kit according to the manufacturer's instructions. An oligonucleotide with the sequence GGGCTCGAGGTTCTTTCCTTGGTAAACAGTGAG was used to prime reverse transcription. The human neuropeptide Y Y_5 receptor cDNA was isolated from SK-N-MC cell cDNA by the PCR. The oligonucleotide primers used in the PCR reaction were GGGGGATCCTGACAAATGTCTTTTTATTCCAAG and GGGCTCGAGGTTCTTTCCTTGGTAAACAGTGAG. The resulting PCR product was ligated into the plasmid pCR3.1. The sequence of this PCR product was identical to nucleotides 20–1418 of the human neuropeptide Y Y_5 receptor cDNA reported by Gerald et al. (1996).

Numerous attempts to express the human neuropeptide Y Y_5 receptor cDNA having the sequence reported by

Gerald et al. (1996) failed. This is probably due to the fact that nucleotides 1–55 of the sequence reported by Gerald et al. (1996) are derived from the human neuropeptide Y Y_5 receptor gene and are not actually found in human neuropeptide Y Y_5 receptor cDNA transcripts (L. Xia and P. VanderVere, unpublished observations; see also Herzog et al., 1997). In contrast, efforts to express both of the rat neuropeptide Y Y_5 receptor cDNA constructs described above were successful. Therefore, a chimeric cDNA incorporating sequences from both the rat and human neuropeptide Y Y_5 receptor cDNAs was constructed. Nucleotides 72–647 of the rat neuropeptide Y Y_5 receptor cDNA sequence reported by Hu et al. (1996) (including the 123 base pair insertion described above) were ligated to nucleotides 462–1418 of the human neuropeptide Y Y_5 receptor cDNA sequence reported by Gerald et al. (1996) at a common *Mun*I restriction site. Subsequently, nucleotides 437 and 576 of the rat neuropeptide Y Y_5 receptor cDNA reported by Hu et al. (1996) were converted from G to C and C to T, respectively, by site directed mutagenesis (Quick Change kit, Stratagene, La Jolla, CA). The resulting chimeric cDNA in the expression vector pcDNA3.1 includes the 5′ untranslated region of the rat neuropeptide Y Y_5 receptor cDNA and encodes a neuropeptide Y Y_5 receptor protein in which amino acids 1–35 are derived from the rat neuropeptide Y Y_5 receptor and amino acids 36–445 are derived from the human neuropeptide Y Y_5 receptor. This chimeric neuropeptide Y Y_5 receptor is designated rhY_5 and is expressed at levels equivalent to the rat neuropeptide Y Y_5 receptor (see below).

2.5. Transfection of CHO and 293 cells

CHO-K1 or 293 cells were transfected by the use of lipofectamine (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Transfected cells were selected by growth in medium supplemented with 400 μg/ml G418 (Life Technologies). G418-resistant colonies were picked via the use of cloning cylinders, expanded and screened for [^{125}I]peptide YY binding (neuropeptide Y Y_1, Y_2 and Y_5 receptors) or [^{125}I]pancreatic polypeptide binding (neuropeptide Y Y_4 receptor) as described below. Cell lines expressing neuropeptide Y receptors were maintained in Ham's F12 medium (CHO-K1 cells) or Dulbecco's modified Eagle's medium (293 cells) supplemented with 10% heat-inactivated fetal calf serum and 200 μg/ml G418. The level of expression of each neuropeptide Y receptor in the CHO or 293 cell lines used in these studies was as follows: human neuropeptide Y Y_1, 893 fmol/mg protein ($n = 2$); rat neuropeptide Y Y_1, 910 fmol/mg protein ($n = 2$); human neuropeptide Y Y_2, 391 fmol/mg protein ($n = 2$); rat neuropeptide Y Y_2, 638 fmol/mg protein ($n = 2$); human neuropeptide Y Y_4, 956 $\pm$ 92 fmol/mg protein ($n = 3$); rat neuropeptide Y Y_4, 421 fmol/mg protein ($n = 2$); rhY_5, 1305 $\pm$ 263 fmol/mg protein ($n = 10$); rat neuropeptide Y Y_5, 1340 fmol/mg protein ($n = 2$).

2.6. Transfection of COS1 cells

COS1 cells were transiently transfected with the mouse neuropeptide Y Y_6 receptor cDNA as described by Parker et al. (1996). The level of expression of the mouse neuropeptide Y Y_6 receptor in the COS1 cell membranes was 681 fmol/mg protein ($n = 2$).

2.7. Preparation of CHO, COS1 or 293 cell membranes

CHO, COS1 or 293 cells were placed on ice and washed once with ice cold phosphate buffered saline. The cells were scraped into membrane buffer (50 mM HEPES [pH 7.3], 0.25 mg/ml Pefabloc [Boehringer Mannheim, Indianapolis, IN], 25 μg/ml leupeptin [Sigma, St. Louis, MO], 25 μg/ml aprotinin [Sigma]), transferred to a Dounce homogenizer and homogenized on ice. The homogenate was centrifuged at 27 000 $\times g$ in a Sorvall SS34 rotor at 4°C and the membrane pellet was resuspended in membrane buffer via homogenization. The homogenate was again centrifuged at 27 000 $\times g$ in a Sorvall SS34 rotor at 4°C and the membrane pellet was resuspended in membrane buffer via homogenization. The membrane homogenate was then stored frozen at −80°C until use. The protein content of the membrane preparations was determined with the BCA protein assay kit (Pierce, Rockford, IL).

2.8. Radioligand binding assays

Binding of [^{125}I] porcine peptide YY (Dupont-NEN, Boston, MA, 2200 Ci/mmol) to the neuropeptide Y receptors expressed in CHO, COS1 or 293 cell membranes was performed in binding buffer (50 mM HEPES [pH 7.3], 0.1% bovine serum albumin, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$). Saturation binding assays were performed in binding buffer containing 0.01–2 nM [^{125}I]peptide YY and 2.5–5 μg of membrane protein (50 μl final volume). Competition binding assays were carried out in binding buffer containing 5–10 μg membrane protein, 0.1 nM [^{125}I]peptide YY and various concentrations of competing peptides (200 μl final volume). In all cases, nonspecific binding was defined as binding in the presence of 1 μM unlabelled human neuropeptide Y. Binding assays were incubated at room temperature for 90 min and were terminated by rapid vacuum filtration through 0.3% polyethyleneimine pretreated glass fiber filters in a 96-well format (Multiscreen FB Filter Plates, Millipore, Bedford, MA or Unifilter-96 GF/C, Packard, Meriden, CT). Each filter was then washed 3 times with 100 μl of phosphate buffered saline and subsequently counted in a gamma

Table 1
Affinity of neuropeptide Y, GR231118, BW1911U90, T-190 and T-241 for cloned human and rat neuropeptide Y receptors expressed in CHO or 293 cells

Peptide	pK_i								
	hY_1	rY_1	hY_2	rY_2	hY_4	rY_4	rhY_5	rY_5	mY_6
Neuropeptide Y	9.4 ± 0.13	9.6 ± 0.086	9.0 ± 0.085	9.4 ± 0.14	7.9 ± 0.21	6.9 ± 0.17	9.1 ± 0.10	9.1 ± 0.14	8.4 ± 0.058
GR231118	10.2 ± 0.088	10.4 ± 0.067	7.2 ± 0.13	7.4 ± 0.14	9.6 ± 0.15	9.7 ± 0.058	7.0 ± 0.17	7.0 ± 0.13	8.8 ± 0.010
BW1911U90	8.3 ± 0.025	8.0 ± 0.17	< 6	< 6	8.3 ± 0.075	6.9 ± 0.10	< 6	< 6	ND
T-190	6.5 ± 0.075	ND	5.5	ND	7.7 ± 0.22	ND	6.1 ± 0.04	ND	ND
T-241	6.8 ± 0.13	ND	6.0 ± 0.11	ND	8.3 ± 0.05	ND	6.7 ± 0.22	ND	ND

All receptors were expressed in CHO-K1 cells with the exception of the rat neuropeptide Y Y_5 receptor which was expressed in 293 cells. pK_i values were determined from radioligand binding data as described in Section 2. All data are expressed as mean ± S.E.M. of data from 3–6 independent experiments. Where the S.E.M. is not indicated, the data shown are the average of two independent experiments. ND, not determined.

counter. All data were analyzed by nonlinear regression analysis (GraphPad Prizm software, San Diego, CA)

2.9. cAMP assays

CHO or 293 cells expressing neuropeptide Y receptors were seeded into 96-well, flat-bottom tissue culture plates at a density of 20 000 cells per well. After approximately 48 h, the cell monolayers were rinsed twice with Hank's balanced salt solution (Life Technologies), then preincubated for 10 min at 37°C with assay buffer (Hank's balanced salt solution supplemented with 4 mM $MgCl_2$, 10 mM HEPES [pH 7.4], 0.2% bovine serum albumin, 1 mM 3-isobutyl-1-methylxanthine [Sigma]). When the an-

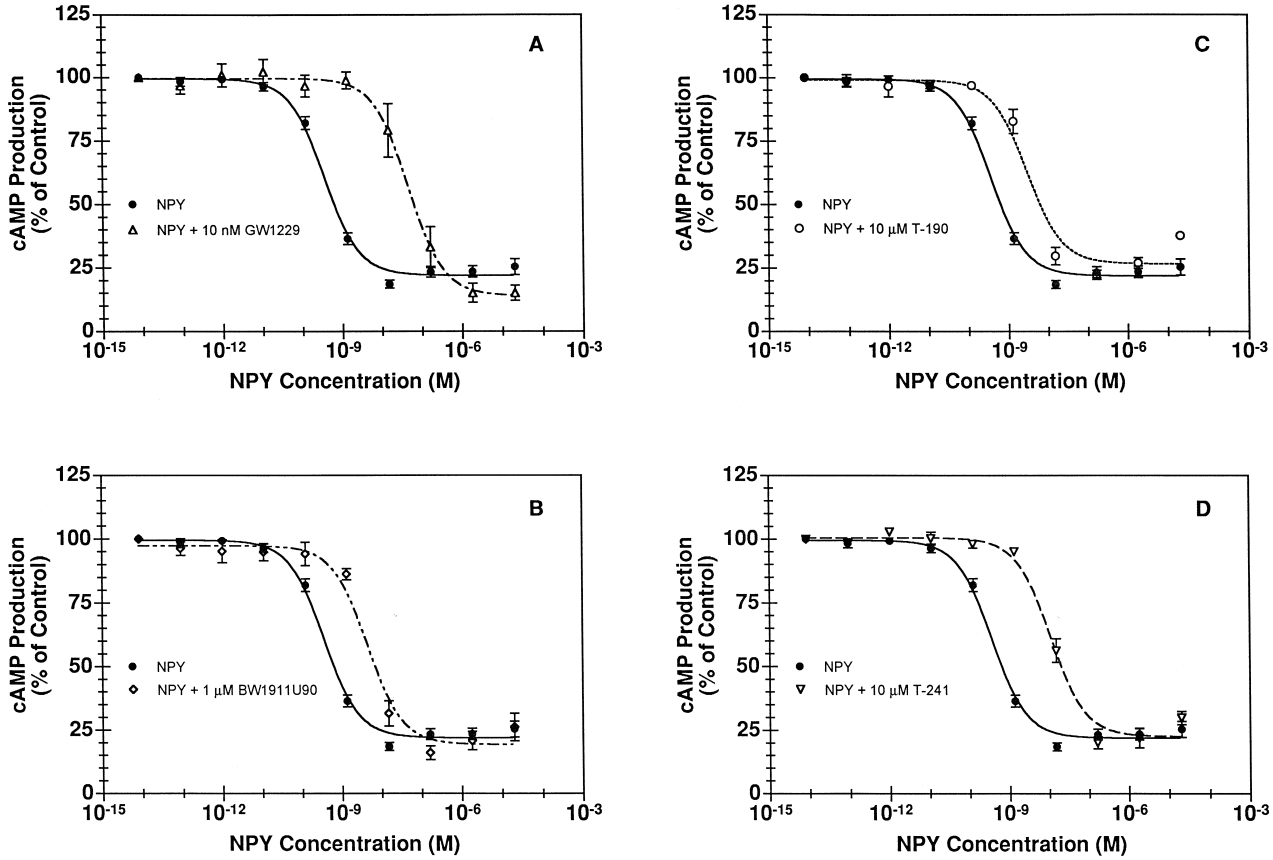

Fig. 2. GR231118, BW1911U90, T-190 and T-241 are competitive antagonists at the human neuropeptide Y Y_1 Receptor. cAMP assays were carried out as described in Section 2. Dose–response curves characterizing the ability of neuropeptide Y to inhibit forskolin-stimulated cAMP formation in CHO cells expressing the human neuropeptide Y Y_1 receptor were carried out in the presence (open symbols) and absence (filled symbols) of 10 nM GR231118 (A), 1 μM BW1911U90 (B), 10 μM T-190 (C) and 10 μM T-241 (D). Basal and forskolin-stimulated cAMP values in these experiments were as follows: A, 2.0 ± 0.3 and 32.7 ± 3.1 pmol/ml; B, 2.4 ± 0.5 and 33.7 ± 3.8 pmol/ml; C, 2.3 ± 0.3 and 34.2 ± 5.5 pmol/ml; D, 2.0 ± 0.1 and 32.9 ± 2.9 pmol/ml. The values shown are the mean ± S.E.M. of data from 3 independent experiments. The apparent pA_2 values calculated from these curves are shown in Table 2.

tagonist effects of peptides were being tested, the peptide antagonists were included in this 10 min preincubation. Subsequently, the assay buffer was removed and replaced with assay buffer containing 1 μM forskolin (Sigma) and various concentrations of peptides. After 10 min at 37°C, the medium was removed and ethanol was added to the cell monolayers. The tissue culture plates were agitated on a platform shaker for 15 min and the plates were then transferred to a warm water bath to evaporate the ethanol. The cell residues were dissolved in FlashPlate assay buffer and the amount of cAMP in each well was quantified using the [^{125}I]cAMP FlashPlate kit (Dupont-NEN) according to the manufacturer's protocol. To calculate EC_{50} values for agonists, all data were analyzed by nonlinear regression analysis (GraphPad Prizm software).

3. Results

In radioligand binding assays, GR231118 has high affinity for the human and rat neuropeptide Y Y_1 and Y_4 receptors and for the mouse neuropeptide Y Y_6 receptor, but has significantly lower affinity for the human and rat neuropeptide Y Y_2 and Y_5 receptors (Table 1). GR231118 does not have any agonist activity at the human or rat neuropeptide Y Y_1 receptors at concentrations up to 10 μM (data not shown). However, GR231118 is a potent competitive antagonist at both the human and rat neuropeptide Y Y_1 receptors (Fig. 2, Table 2). GR231118 is also a potent agonist at the human neuropeptide Y Y_4 receptor (Fig. 3, Table 2). GR231118 has weak agonist activity at the neuropeptide Y Y_2 and Y_5 receptors (Fig. 4, Table 2).

BW1911U90, T-190 and T-241 have a qualitatively similar pharmacological profile to GR231118, although these peptides are significantly less potent. In radioligand binding assays, BW1911U90 has high affinity for the human and rat neuropeptide Y Y_1 and Y_4 receptors, but has negligible affinity for the human and rat neuropeptide Y Y_2 and Y_5 receptors (Table 1). Interestingly, the affinity of BW1911U90 for the rat neuropeptide Y Y_4 receptor is significantly less than its affinity for the human neuropeptide Y Y_4 receptor. This is consistent with the significantly different pharmacological properties of the human and rat neuropeptide Y Y_4 receptors that have been previously documented (e.g., Walker et al., 1997). T-190 and T-241 have high affinity for the human neuropeptide Y Y_4 receptor, but have only modest affinity for the human neuropeptide Y Y_1, Y_2 and Y_5 receptors (Table 1). Neither BW1911U90, T-190 nor T-241 have agonist activity at the human neuropeptide Y Y_1 receptor at concentrations up to 10 μM (data not shown). However, all three peptides are moderately potent competitive antagonists at the human neuropeptide Y Y_1 receptor (Fig. 2, Table 2). Like GR231118, BW1911U90, T-190 and T-241 are agonists at the human neuropeptide Y Y_4 receptor, although these

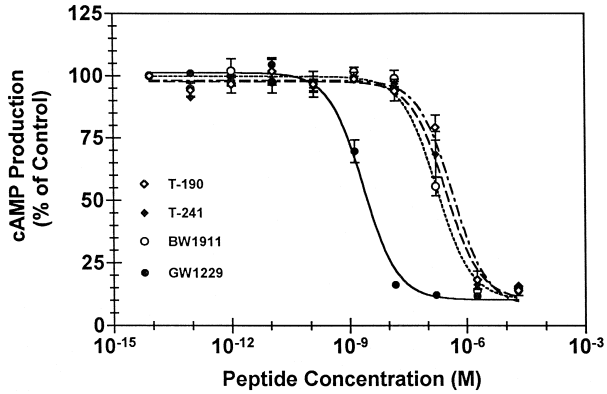

Fig. 3. GR231118, BW1911U90, T-190 and T-241 are human neuropeptide Y Y_4 receptor agonists. cAMP assays were carried out as described in Section 2. The figure depicts dose-response curves characterizing the ability of GR231118 (●), BW1911U90 (○), T-190 (◇) and T-241 (◆) to activate the human neuropeptide Y Y_4 receptor. The basal and forskolin-stimulated cAMP values in these experiments were 1.7 ± 0.1 and 21.7 ± 2.3 pmol/ml. The values shown are the mean $\pm$ S.E.M. of data from 3 independent experiments. pEC_{50} values determined in these experiments are shown in Table 2.

Table 2
Agonist or antagonist potency of neuropeptide Y, GR231118, BW1911U90, T-190 and T-241 for cloned human and rat neuropeptide Y receptors expressed in CHO or 293 cells

Peptide	Apparent pA_2 or pEC_{50}					
	hY_1	rY_1	rY_2	hY_4	rhY_5	rY_5
Neuropeptide Y	9.4 ± 0.05	9.5 ± 0.68	8.7 ± 0.10	6.6 ± 0.16	8.4 ± 0.07	9.0 ± 0.03
GR231118	**10.5 ± 0.21**	**10.0 ± 0.39**	6.0 ± 0.09	8.6 ± 0.10	5.7	6.1 ± 0.02
BW1911U90	**7.1 ± 0.049**	ND	ND	6.8 ± 0.03	ND	
T-190	**5.8 ± 0.08**	ND	ND	6.3 ± 0.07	ND	
T-241	**6.5 ± 0.03**	ND	ND	6.6 ± 0.08	ND	

All receptors were expressed in CHO-K1 cells with the exception of the rat neuropeptide Y Y_5 receptor which was expressed in 293 cells. Apparent pA_2 values (shown in bold) were determined from dose ratio analysis of neuropeptide Y concentration response curves in the presence and absence of competitive antagonist as described (Kenakin, 1993). pEC_{50} values (shown in normal text) were calculated as described in Section 2. In all cases where the peptides behaved as agonists, the intrinsic activity of the peptides was equal to that of neuropeptide Y. The values shown are the mean $\pm$ S.E.M. of data from 3–23 independent experiments. Where S.E.M. values are not shown, the data represent the average value from two independent experiments. ND, not determined.

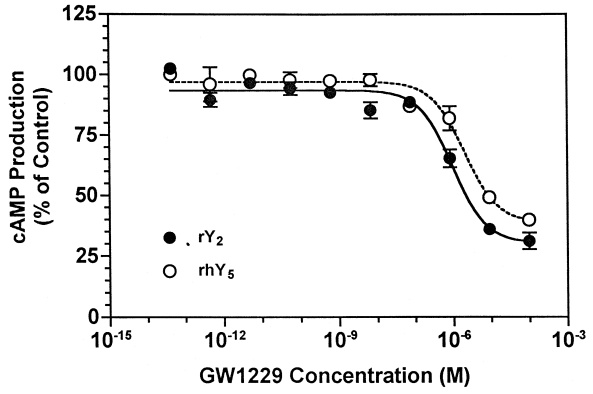

Fig. 4. GR231118 is a weak agonist at the neuropeptide Y Y_2 and Y_5 receptors. cAMP assays were carried out as described in Section 2. The figure depicts dose-response curves characterizing the ability of GR231118 to activate the rat neuropeptide Y Y_2 (●) and rhY_5 (○) receptors. The basal and forskolin-stimulated cAMP values in these experiments were 1.8 ± 0.3 and 14.8 ± 4.1 pmol/ml (rat neuropeptide Y Y_2 receptor) and 2.5 ± 0.2 and 21.2 ± 8.7 pmol/ml (rhY_5 receptor). The values shown are the mean ± S.E.M. (rat neuropeptide Y Y_2 receptor) of data from three independent experiments or mean ± range (rhY_5 receptor) of data from two independent experiments. pEC_{50} values determined in these experiments are shown in Table 2.

three peptides are significantly less potent than GR231118 (Fig. 3, Table 2).

4. Discussion

GR231118 has been reported to be a potent neuropeptide Y Y_1 receptor antagonist with little activity at the neuropeptide Y Y_2 receptor (Hegde et al., 1995; Daniels et al., 1995). Because of its very high potency and selectivity, GR231118 has been used to determine the role of the neuropeptide Y Y_1 receptor in mediating various physiological effects of neuropeptide Y (Kanatani et al., 1996; Hegde et al., 1995; Lew et al., 1996). The present study demonstrates that GR231118 is not only a potent neuropeptide Y Y_1 receptor antagonist, but is also a potent neuropeptide Y Y_4 receptor agonist. GR231118 also has high affinity for the mouse neuropeptide Y Y_6 receptor, although its functional activity at this receptor has not been determined. GR231118 has weak agonist activity at the neuropeptide Y Y_2 and Y_5 receptors. GR231118 is 20- to 60-fold more potent in blocking the neuropeptide Y Y_1 receptor than in activating the neuropeptide Y Y_4 receptor or in binding to the neuropeptide Y Y_6 receptor. Thus, GR231118 can be considered to be a relatively selective neuropeptide Y Y_1 receptor antagonist in experiments where its concentration can be precisely defined (e.g., isolated tissue experiments). However, the concentration of GR231118 cannot always be precisely defined, particularly in studies in which GR231118 is administered directly into the central nervous system (e.g., Kanatani et al., 1996). Interpretation of data obtained in such studies requires that the activity of GR231118 at the neuropeptide Y Y_1, Y_4, and Y_6 receptors be taken into consideration. Because the physiological roles of the neuropeptide Y Y_4 and Y_6 receptors have not yet been determined, it is not clear how much the activity of GR231118 at these receptors detracts from its utility as a neuropeptide Y Y_1 receptor antagonist. In practice, neuropeptide Y Y_1 receptor antagonism may be distinguished from neuropeptide Y Y_4 receptor agonism by determining if GR231118 mimics or antagonizes the effects of neuropeptide Y.

BW1911U90 has been reported to be potent neuropeptide Y Y_1 receptor antagonist with little activity at the neuropeptide Y Y_2 receptor (Leban et al., 1995). BW1911U90 is inactive at the neuropeptide Y Y_2 and Y_5 receptors (Table 2). However, the potency of BW1911U90 in activating the neuropeptide Y Y_4 receptor is similar to its potency in antagonizing the neuropeptide Y Y_1 receptor (Table 2). Therefore, both the neuropeptide Y Y_4 agonist and neuropeptide Y Y_1 antagonist activity of BW1911U90 must be taken into consideration when interpreting pharmacological studies that use this peptide to characterize the receptors mediating various physiological effects of neuropeptide Y.

T-190 and T-241 have been reported to be potent neuropeptide Y Y_1 receptor antagonists with little activity at the neuropeptide Y Y_2 receptor (Balasubramaniam et al., 1996a,b). As was seen with GR231118 and BW1911U91, T-190 and T-241 were neuropeptide Y Y_1 receptor antagonists and neuropeptide Y Y_4 receptor agonists (Table 2). However, T-190 and T-241 do not have particularly high affinity or potency for any of the cloned neuropeptide Y receptors (Tables 1 and 2). Hence, the utility of these peptides in the pharmacological characterization of the known neuropeptide Y receptors is limited (but see below).

In some cases, the peptides tested in this study are more potent in radioligand binding assays than in functional assays (cf. Tables 1 and 2). These discrepancies may be due to a number of factors. The radioligand binding and cAMP assays are performed under different conditions (different buffers, different temperatures, whole cells vs. cell membranes). Furthermore, radioligand binding assays with agonist radioligands may measure primarily the high affinity, G protein-coupled state of the receptor whereas the functional assay may measure predominantly the low affinity, uncoupled state of the receptor (DeLean et al., 1980). Hence, full and partial agonists that induce substantial receptor G protein coupling may appear more potent in binding assays than in functional assays. Similar discrepancies between affinities of peptides for NPY receptors in radioligand binding and functional assays have been frequently reported in the literature, particularly for the Y_4 receptor (e.g., Lundell et al., 1995). Therefore, pharmacological characterization of peptides at NPY receptors ideally requires determination of the potencies of the peptides in both radioligand binding and functional assays.

GR231118, BW1911U90, T-190 and T-241 are all analogues of the C-terminus of neuropeptide Y and are all neuropeptide Y Y_1 receptor antagonists and neuropeptide Y Y_4 receptor agonists. This common pharmacological profile suggests that the C-terminus of neuropeptide Y (and the related peptides peptide YY and pancreatic polypeptide) is sufficient to activate the neuropeptide Y Y_4 receptor, but is not sufficient to activate the neuropeptide Y Y_1 receptor. This conclusion is consistent with previous data that indicate that the recognition and activation of the neuropeptide Y Y_1 receptor requires amino acids in both the N-terminus and C-terminus of the peptide (Beck-Sickinger and Jung, 1995) whereas the C-terminus of neuropeptide Y is sufficient for recognition and activation of the neuropeptide Y Y_4 receptor (Gehlert et al., 1996; Walker et al., 1997). Recently, this view has been questioned by Leban et al., who synthesized peptide analogues of the C-terminus of neuropeptide Y that are potent neuropeptide Y Y_1 receptor agonists (Leban et al., 1995). However, the peptides synthesized by Leban et al. (1995) may actually mimic both the N-terminus and C-terminus of neuropeptide Y (see discussion in Daniels et al., 1997). Thus, continued design of analogues of the C-terminus of neuropeptide Y should be a reasonable strategy for the identification of neuropeptide Y Y_1 receptor antagonists and neuropeptide Y Y_4 receptor agonists. The challenge, however, will be to develop strategies to separate the neuropeptide Y Y_1 antagonist activity and neuropeptide Y Y_4 agonist activity in peptide analogues of the C-terminus of neuropeptide Y.

Interestingly, BW1911U90, T-190 and T-241 are significantly less potent at the cloned human neuropeptide Y Y_1 receptor expressed in CHO cells than at the neuropeptide Y receptor expressed in human erythroleukemia cells. The potency of BW1911U90 in antagonizing neuropeptide Y-induced increases in cytosolic calcium in human erythroleukemia cells ($pA_2 \sim 8.6$; Leban et al., 1995) is higher than the potency of this peptide in antagonizing neuropeptide Y-induced decreases in cAMP formation in CHO cells expressing the cloned human neuropeptide Y Y_1 receptor ($pA_2 = 7.13$; Table 2). Similarly, the reported potency of T-190 and T-241 to block functional effects of neuropeptide Y in human erythroleukemia cells (T-190 $pA_2 = 8.4$ Balasubramaniam et al., 1996a; T-241 $pIC_{50} = 8.8$; Tao et al., 1997) is also much higher than the potency of these peptides to block functional effects of neuropeptide Y in CHO cells expressing the cloned human neuropeptide Y Y_1 receptor (T-190 $pA_2 = 5.8$, T-241 $pA_2 = 6.5$; Table 2). While human erythroleukemia cells are thought to express only the neuropeptide Y Y_1 receptor (Feth et al., 1992), these data may indicate that these cell lines express a novel neuropeptide Y receptor with high affinity for BW1911U90, T-190 and T-241. In support of this notion, the potency of T-190 in inhibiting isoproterenol-stimulated cAMP formation in SK-N-MC cells ($pIC_{50} = 6.6$ Balasubramaniam et al., 1996a), a human neuroblastoma cell line known to express the neuropeptide Y Y_1 receptor, is similar to the potency of this peptide at the cloned human neuropeptide Y Y_1 receptor reported in this study (Table 2). Further pharmacological characterization of the neuropeptide Y receptor in human erythroleukemia cells is warranted to conclusively determine if this cell line expresses a novel neuropeptide Y receptor subtype.

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