Pharmacological Characterization of $^{125}$I-1229U91 Binding to Y1 and Y4 Neuropeptide Y/Peptide YY Receptors

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ABSTRACT

1229U91 (GW1229 or GR231118) [Ile,Glu,Pro,Dpr,Tyr,Arg,Leu,Arg,Tyr-NH$_2$]$_2$ cyclic (2,4’),(2,4’)-diamide has been reported by several research groups to be a potent antagonist at the Y1 neuropeptide Y (NPY) receptor subtype. However, 1229U91 also displaces $^{125}$I-peptide YY (PYY) with high affinity from the Y4 subtype. Previously, we reported that 1229U91 had full agonist properties for the Y4 receptors. To characterize the pharmacological properties of 1229U91 directly, we had it radiiodinated with the chloromine-T method. $^{125}$I-1229U91 binds to cell lines expressing the human Y1 and Y4 receptors with high affinity. The $K_a$ and $B_{max}$ for $^{125}$I-1229U91 binding to Y1 were 14.9 pM and 1458 fmol/mg protein, respectively. The Y4 receptor $B_{max}$ of $^{125}$I-1229U91 with a $K_a$ of 12.5 pM and a $B_{max}$ of 1442 fmol/mg protein. When competing $^{125}$I-1229U91 binding from Y1 and Y4 receptors, a similar rank order of potency was observed: 1229U91 > [Leu$^{31}$,Pro$^{34}$]-NPY ≥ [Leu$^{31}$,Pro$^{34}$]-PYY > PYY ≥ NPY > NPY(2–36) > PYY(3–36). Pancreatic polypeptide (PP) potently displaced $^{125}$I-1229U91 from the Y4 receptor, but displayed little affinity for Y1. In autoradiographic studies with rat brain sections, $^{125}$I-1229U91 bound with a distribution similar to that reported for the Y1 receptor when localized with $^{125}$I-[Leu$^{31}$,Pro$^{34}$]-PYY. Brain regions exhibiting binding sites for $^{125}$I-PP were not detected with this radioligand. Those include the interpeduncular nucleus and the periventricular nucleus of the hypothalamus. Furthermore, $^{125}$I-labeled rat PP was not displaced from these areas with 10 nM 1229U91. Thus, $^{125}$I-1229U91 is a high affinity Y1 and Y4 radioligand and binds with a distribution in the rat brain consistent with the localization of the Y1 receptor.

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Since the discovery (Tatemoto, 1982) of neuropeptide Y (NPY), its receptor has been the focus of research for potential antagonist design and drug discovery. A novel peptide antagonist 1229U91 [Ile,Glu,Pro,Dpr,Tyr,Arg,Leu,Arg,Tyr-NH$_2$]$_2$ cyclic (2,4’),(2,4’)-diamide] was synthesized based on the C terminus of NPY (Daniels et al., 1995) that displaced $^{125}$I-NPY (Daniels et al., 1995) and $^{125}$I-peptide YY (PYY) (Kanatani et al., 1995) with high affinity from the human Y1 receptor containing cell line SK-N-MC. Furthermore, the displacement was competitive in nature, and the compound was reported to be a pure antagonist at the Y1 receptor found in human erythroblastic cells (Daniels et al., 1995). The selectivity of 1229U91, however, remains somewhat controversial. In rat brain homogenates, 1229U91 displaced $^3$H-NPY with 10-fold higher affinity than the Y1 containing SK-N-MC cells (Daniels et al., 1995). From their findings, these investigators concluded that 1229U91 did not appear to be selective for any particular NPY receptor subtype. In contrast, other studies (Hegde et al., 1995; Kanatani et al., 1996) have reported that 1229U91 selectively inhibited $^{125}$I-PYY binding to the Y1 receptor with SK-N-MC cells. To clarify 1229U91 selectivity, we evaluated it with the Y1 containing SK-N-MC and clonal cell lines containing Y2, Y4, and Y5 receptor subtypes (Schober et al., 1998). We found that not only did 1229U91 displace $^{125}$I-PYY with high affinity for the Y1 but also Y4 receptors. Therefore, we concluded that 1229U91 was a nonselective inhibitor of NPY binding. Subsequent work was performed to look at functional activity of 1229U91 at the Y1 and Y4 receptors. With adenylate cyclase assays, we (Schober et al., 1998) and others (Parker et al., 1998) found that 1229U91 was a potent antagonist at the Y1 receptor but an equally potent agonist at the Y4 receptor.

To better understand the pharmacology of 1229U91, a radiolabeled version of the molecule was necessary. In the present study, the binding of $^{125}$I-1229U91 to the cloned Y1 and Y4 receptors was evaluated. In addition, autoradiographic studies were performed to investigate the distribution of $^{125}$I-1229U91 throughout the rostral-caudal extent of the rat brain.

ABBREVIATIONS: NPY, neuropeptide Y; 1229U91, [Ile,Glu,Pro,Dpr,Tyr,Arg,Leu,Arg,Tyr-NH$_2$]$_2$ cyclic (2,4’),(2,4’)-diamide] PYY, peptide YY; rPP, rat pancreatic polypeptide; pPYY, porcine PYY; CHO, Chinese hamster ovary; bPP, bovine pancreatic polypeptide; hPP, human pancreatic polypeptide; IPN, interpeduncular nucleus.
Materials and Methods

Cell Culture. Cells stably expressing human Y1 (Gehlert et al., 1996b), Y4 (Lundell et al., 1995), and rat Y1 (Eva et al., 1990) receptors were grown in T-150 flasks containing Dulbecco's minimal essential media with 5% fetal calf serum (Life Technologies, Gaithersburg, MD). The flasks were placed in a humidified incubator at 37°C containing 5% CO₂. The confluent cells were removed manually from the flasks by scraping. Cells were then washed with PBS, pelleted by centrifugation, and stored at −70°C until assayed.

Rat Brain Tissue Preparation. Male, 250- to 350-g Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were anesthetized with halothane and rapidly decapitated. The brains were quickly removed and placed on ice. The tissues were homogenized in 50 mM Tris (pH 7.4) with a Polytron (Brinkmann Instruments, Westbury, NY) with three 10-s bursts. After an initial spin at 800g for 10 min, pelleting the supernatant at 35,000g for an additional 20 min isolated membranes. Membranes were stored at −70°C until assayed.

Homogenate-Binding Studies. Binding assays were conducted as previously described (Gehlert et al., 1992) with isolated crude membrane homogenates. The cell pellets were resuspended with a Polytron homogenizer (Brinkmann) in 25 mM HEPES (pH 7.4) buffer containing 2.5 mM CaCl₂, 1.0 mM MgCl₂, and 2 g/l bacitracin. Incubations were performed for 2 h at room temperature in a final volume of 200μl containing known amounts of 125I-1229U91 or 125I-porcine PYY (pPYY) (specific activity 2200 Ci/mM; NEN, Boston, MA). In the saturation experiments, 12 different concentrations of 125I-1229U91 were used. A 96-well cell harvester (Tomtec, Orange, CA) was used to terminate the incubations by rapid filtration through GF/C filters (Wallac, Gaithersburg, MD) presoaked in 0.3% polyethyleneimine (Sigma Chemical Co., St. Louis, MO). After a 5-ml wash with ice-cold 50 mM Tris (pH 7.4) with a Polytron (Brinkmann Instruments, Westbury, NY) with three 10-s bursts. After an initial spin at 800g for 10 min, pelleting the supernatant at 35,000g for an additional 20 min isolated membranes. Membranes were stored at −70°C until assayed.

 Autoradiographic Studies. Male, 250- to 350-g Sprague-Dawley rats were anesthetized with halothane and decapitated. The brains were rapidly removed and stored at −70°C. The brains were mounted on chucks and sectioned at 12 μm with a cryostat (Hacker, Fairfield, NJ). Coronal sections were thaw mounted onto gelatin-coated slides, placed at −20°C overnight, and stored at −70°C until assayed. Sections were incubated according to a previously described protocol (Gehlert et al., 1992) with slight modifications. Sections were initially preincubated for 30 min in Krebs-Ringer buffer containing 0.4% BSA and 0.5% bacitracin. The sections were then placed into glass jars either containing 100 μM 125I-1229U91 or 25 μM 125I-rat pancreatic polypeptide (rPP) (specific activity 2200 Ci/mM; NEN, Boston, MA). Non-specific binding was defined by incubating adjacent sections with the addition of 1 μM 1229U91 or 10 nM rat pancreatic polypeptide (PP). Following a 2-h incubation, the sections were rinsed four times in fresh buffer without BSA for 5 min each and dried rapidly. The labeled sections were exposed for 3 or 7 days to Hyperfilm β-max (Amersham, Arlington Heights, IL) for 125I-1229U91 or 125I-PP, respectively. The films were developed in D-19 developer (Kodak, Rochester, NY) for 5 min.

Results

Saturation-Binding Analysis of 125I-1229U91 to Membranes Prepared from Rat Brains and Cell Lines Expressing Human Y1 and Y4 Receptors. The affinity of 125I-1229U91 binding to both the Y1 and Y4 receptors, as well as the rat forebrain membranes was examined. Figure 1 illustrates a saturation isotherm for the human Y1 receptor stably expressed in an AV12 cell line. Nonlinear regression analysis of the specific binding revealed a single (r² = 0.97) saturable high-affinity site with a Kd = 14.9 ± 1.5 pM and a Bmax = 1458 ± 43 fmol/mg protein. In Fig. 2, 125I-1229U91 binding to the human Y4 receptor stably expressed in a Chinese hamster ovary (CHO) cell line is illustrated. A single (r² = 0.97) saturable high-affinity site was also observed and had a Kd = 12.5 ± 1.1 pM and a Bmax = 1442 ± 35 fmol/mg protein. To characterize the binding to rat brains, similar saturation studies were performed (Fig. 3). In this case, 125I-1229U91 also bound to a single (r² = 0.99) saturable site with a Kd = 136 ± 6 pM and a Bmax = 549 ± 8 fmol/mg protein. No specific binding was observed to cell lines expressing the Y2 and Y5 receptor subtypes (data not shown).

Pharmacology of 125I-1229U91 and 125I-pPYY Binding to Rat Brain and Cell Lines Expressing Y1 and Y4 Receptors. Because 125I-1229U91 bound with similar affinity to the human Y1 and Y4 containing cell lines, the pharmacology of 125I-1229U91 binding to their respective cell lines was examined. These data are presented in Table 1. When competing 10 pM 125I-1229U91 binding from human Y1 (Fig. 4) and Y4 (Fig. 5) receptors with various peptides and peptide analogs, a similar rank order of potency was observed: 1229U91 > [Leu³¹, Pro³⁴⁻]NPY ≥ [Leu³¹, Pro³⁴⁻]PYY > PYY ≥ NPY > NPY(2–36) > PYY(3–36). The displacement curves were best fit to a one-site model. Human PP inhibited with high-affinity 125I-1229U91 binding to the
The Y1 NPY receptor subtype was the first member of the PP-fold peptide receptor family to be cloned (Herszog et al., 1992; Larhammar et al., 1992). This subtype displays high affinity for both PYY and NPY with little affinity for PP (Herszog et al., 1992; Larhammar et al., 1992). In contrast, the second receptor cloned from the family of PP-fold peptide receptors, the Y4/PP1, has high affinity for PP and lower affinity for PYY and NPY (Bard et al., 1995; Lundell et al., 1995). With expression-cloning techniques, the Y2 receptor subtype was the next receptor cloned in this receptor family (Gerald et al., 1995; Gehlert et al., 1996a). Unlike the Y1 subtype, the Y2 receptor binds C-terminal fragments of NPY/PYY as well as intact NPY and PYY. A novel receptor from the rat hypothalamus was the fourth NPY receptor subtype cloned and was designated the Y5 (Gerald et al., 1996). This was the first receptor that appeared to have a pharmacological profile that correlates with in vivo feeding studies. The Y5 subtype binds NPY and PYY, C-terminal fragments of NPY and PYY, and Pro34-substituted analogs of NPY and PYY as well as PP. One distinguishing feature of the Y5 receptor is its high affinity for n-Trp32]NPY. In another study, a novel NPY receptor was cloned and expressed from a mouse genomic cDNA library (Weinberg et al., 1996). Also designated the Y5, the pharmacology of this novel receptor resembles that of the Y1 subtype and is distinct from that described for the Y2, Y3, and Y4 receptors. Referred to as both Y5 and/or Y2b and/or PP2, this murine receptor has now been designated y6 by the IUPHAR organization (Burkhoff et al., 1998). The y6 sequence does not encode a functional receptor in either the rat or human (Gregor et al., 1996).

The bridged antiparallel dipeptide compound 1229U91 was first described as a potent NPY receptor antagonist. Subsequently, it was found to be selective for Y1 over Y2 receptors. The high potency and metabolic stability of this peptide indicated that a radioiodinated version would make a suitable radioligand. In the present study, we have used 125I-1229U91 binding to the human Y1 receptor with a $K_i$ of 34.5 ± 6.6 pM, whereas the rat Y1 receptor had a $K_i$ of 131.9 ± 22 pM.

**Discussion**

The Y1 NPY receptor subtype was the first member of the PP-fold peptide receptor family to be cloned (Herszog et al., 1992; Larhammar et al., 1992). This subtype displays high affinity for both PYY and NPY with little affinity for PP (Herszog et al., 1992; Larhammar et al., 1992). In contrast, the second receptor cloned from the family of PP-fold peptide receptors, the Y4/PP1, has high affinity for PP and lower affinity for PYY and NPY (Bard et al., 1995; Lundell et al., 1995). With expression-cloning techniques, the Y2 receptor subtype was the next receptor cloned in this receptor family (Gerald et al., 1995; Gehlert et al., 1996a). Unlike the Y1 subtype, the Y2 receptor binds C-terminal fragments of NPY/PYY as well as intact NPY and PYY. A novel receptor from the rat hypothalamus was the fourth NPY/PP1 receptor subtype cloned and was designated the Y5 (Gerald et al., 1996). This was the first receptor that appeared to have a pharmacological profile that correlates with in vivo feeding studies. The Y5 subtype binds NPY and PYY, C-terminal fragments of NPY and PYY, and Pro34-substituted analogs of NPY and PYY as well as PP. One distinguishing feature of the Y5 receptor is its high affinity for n-Trp32]NPY. In another study, a novel NPY receptor was cloned and expressed from a mouse genomic cDNA library (Weinberg et al., 1996). Also designated the Y5, the pharmacology of this novel receptor resembles that of the Y1 subtype and is distinct from that described for the Y2, Y3, and Y4 receptors. Referred to as both Y5 and/or Y2b and/or PP2, this murine receptor has now been designated y6 by the IUPHAR organization (Burkhoff et al., 1998). The y6 sequence does not encode a functional receptor in either the rat or human (Gregor et al., 1996).
Furthermore, the distribution of 125I-1229U91 binding is with a pharmacological profile consistent for the Y1 receptor. tani et al., 1996) and forebrain (present study) membranes species differences between the rat and human receptors in the human Y1 or Y4 receptors. This suggests there may be lower affinity compared with the cell lines expressing either however, 125I-1229U91 in the rat brain had a significantly lower affinity compared with the cell lines expressing either the human Y1 or Y4 receptors. This suggests there may be species differences between the rat and human receptors in the potency for 1229U91. In this study, the K_i values for 1229U91 in both the rat brain and the cloned rat Y1 receptor were similar to the K_i value obtained for 125I-1229U91 in the rat forebrain. The K_i and K_d values for 1229U91 and 125I-1229U91, respectively, at the human Y1 receptor were also similar, but, interestingly, 1229U91 was a log order more potent at the human receptor than the rat. Species differences in the potency of 1229U91 also were noted in a study by Parker et al. (1998). There are several lines of evidence that suggest another PP-fold peptide receptor subtype may exist in the rat brain. First, at concentrations up to 1 μM, both NPY and PYY will not inhibit 125I-bPP from the area postrema (Whitcomb et al., 1990). This differs substantially from the pharmacology described for the Y1, Y2, Y4, and Y5 receptor subtypes. Secondly, Schwartz et al. (1987) demonstrated that Schwartz et al., 1993 demonstrated that 1229U91 was also a potent agonist for the Y4 receptor. As expected, 125I-1229U91 was displaced from rat hypothalamic (Kanata et al., 1996) and forebrain (present study) membranes containing the human Y1 receptor. Various peptides or peptide analogs were incubated for 2 h at room temperature (PP; PYY; [Leu31,Pro34]-PYY; [Leu31,Pro34]-NPY; [Leu31,Pro34]-NPY; [Leu31,Pro34]-NPY, PYY(3–36); [Leu31,Pro34]-NPY, PYY(2–36); 1229U91). Data are expressed as a percentage of specific binding and were obtained from four experiments performed in duplicate. Nonspecific binding was defined as the binding remaining in the presence of 1 μM 1229U91.
incubation was carried out in the presence of 100 pM 125I-pPYY for 2 h. [Leu31,Pro34]-NPY; f Y1 receptors. The
affinity of 1229U91 was evaluated at the rat (M NPY.

m M 1229U91. The incubation was carried out over a 2-h period in the presence of 140 pM 125I-1229U91. Data are expressed as a percentage of specific binding and were obtained from four experiments performed in duplicate. Nonspecific binding was defined as the binding remaining in the presence of 1 μM 1229U91.

investigators also noted that 125I-h/rPP-labeled sites were found in the interpeduncular nucleus (IPN), and the paraventricular nucleus in the rat brain. The rank order of potency they observed to displace 125I-h/rPP binding from the rat brain was PP > [Leu31,Pro34]-PYY > PYY > NPY > PYY(3–36) > BIBP3226. In agreement with these results, we observed an identical rank order of potency for the cloned human Y4 receptor in this study. Therefore, the Y4 subtype would be a likely candidate for the receptor labeled by 125I-h/rPP in the rat brain. However, radiiodinated bovine (Whitcomb et al., 1990; Gehlert et al., 1997), rat, and human (Trinh et al., 1996; present study) PP bound to a high density of sites in the rat IPN. We speculate that this region may contain an “atypical site” because only PP displaced 125I-rPP from the IPN. Neither 1229U91 that has high affinity for the}

Y1 and Y4 receptors (Schober et al., 1998) nor PYY(3–36) that has high affinity for the Y2 and Y5 (Gerald et al., 1996) receptors displaced the IPN-binding sites. The peptide analog [Leu31,Pro34]-PYY slightly inhibited 125I-1PP binding from the IPN. In addition, this receptor uniquely possesses high affinity for 125I-1PP because both 125I-1229U91 (present study) and 125I-[Leu31,Pro34]-PYY (Gehlert et al., 1997) did not exhibit detectable binding to the rat IPN. Thus, these data are suggestive that the cloned Y4 receptor subtype is not the same receptor identified by radiolabeled PP in the rat IPN.

in conclusion, we have examined the pharmacology and binding of 125I-1229U91 to cloned NPY Y1 and Y4 receptors and the rat brain. This radioligand bound with high affinity to the both the human Y1 and Y4 receptors. In rat brain, the binding pharmacology and distribution was consistent with the Y1 subtype. However, 125I-1229U91 had significantly lower affinity for rat Y1 receptors compared with the cell lines expressing either the human Y1 or Y4 receptors, suggesting species differences in the potency for 1229U91. Thus, 125I-1229U91 is a useful antagonist ligand for the study of Y1 receptors. These results provide evidence that, 125I-PP binding to the interpeduncular nucleus occurs to a non-Y4 receptor. We speculate that the IPN may contain a unique NPY receptor subtype.

References

Fig. 9. Autoradiographic localization of $^{125}$I-rPP binding sites through coronal sections containing the interpeduncular nucleus. Rat brain sections were incubated with 25 pM $^{125}$I-rPP as described in Materials and Methods. An additional section was incubated with 10 nM rPP to define nonspecific binding. A representative autoradiogram for nonspecific binding is presented in E. Autoradiograms were obtained by exposing the radiolabeled sections to X-ray film for 7 days.


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