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The neuropeptide Y Y_1 receptor selective radioligand, [^{125}I][Leu^{31},Pro^{34}]peptide YY, is also a high affinity radioligand for human pancreatic polypeptide 1 receptors

Donald R. Gehlert [a,*], Susan L. Gackenheimer [a], Douglas A. Schober [a], Lisa Beavers [a], Robert Gadski [a], J. Paul Burnett [a], Nancy Mayne [a], Ingrid Lundell [b], Dan Larhammar [b]

[a] *Central Nervous System and Endocrine Research, Mail Code 0510, Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA*

[b] *Department of Medical Pharmacology, Uppsala University, Box 593, S-751 24 Uppsala, Sweden*

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Abstract

A number of receptors for the pancreatic polypeptide-fold peptides are proposed based on findings from pharmacology and molecular biology studies. Neuropeptide Y and peptide YY have similar affinity for neuropeptide Y Y_1 and neuropeptide Y Y_2 while pancreatic polypeptide has highest affinity for pancreatic polypeptide 1. Pro^{34}-substituted analogs of neuropeptide Y and peptide YY have selectivity for neuropeptide Y Y_1 over neuropeptide Y Y_2 receptors. In the present study, we found that one such 'neuropeptide Y Y_1-selective' radioligand, [^{125}I][Leu^{31},Pro^{34}]peptide YY, also binds with high affinity to the pancreatic polypeptide 1 receptor. Therefore, caution needs to be exercised when using Pro^{34}-analogs to define the neuropeptide Y Y_1 receptor in vivo and using tissue preparations.

Keywords: Neuropeptide Y; Pancreatic polypeptide; Peptide YY; [Leu^{31},Pro^{34}]Peptide YY

1. Introduction

Neuropeptide Y is a 36-amino-acid peptide that belongs to the pancreatic polypeptide-fold family of peptides. This family also includes the endocrine peptides, peptide YY and pancreatic polypeptide. Presently, there are at least four proposed receptors for the pancreatic polypeptide-fold peptide family (for review see Gehlert, 1994). The neuropeptide Y Y_1 receptor is believed to be mainly postsynaptically localized in the peripheral nervous system but it may also be prejunctional in selected tissues, e.g. rabbit vas deferens (Doods and Krause, 1991). The neuropeptide Y Y_2 receptor may be localized both pre- and postsynaptically. Both neuropeptide Y Y_1 and neuropeptide Y Y_2 receptors are found in the central nervous system and have unique neuroanatomical distributions. A third neuropeptide Y receptor (neuropeptide Y Y_3) has been reported as having a higher affinity for neuropeptide Y than peptide YY and can be found in the brainstem and adrenal. A recently discovered fourth receptor for this peptide family is the pancreatic polypeptide 1 receptor. The human pancreatic polypeptide 1 receptor has highest affinity for pancreatic polypeptide with lower affinity for peptide YY and substantially lower affinity for neuropeptide Y (Lundell et al., 1995). Also, this receptor has been cloned by another group and given the designation 'Y_4' (Bard et al., 1995). Other proposed receptors include a hypothalamic 'feeding' receptor that responds to neuropeptide Y and peptide YY and a peptide YY-preferring receptor (Gehlert, 1994; Gehlert and Hipskind, 1995).

The neuropeptide Y Y_1 receptor has low affinity for C-terminal fragments of neuropeptide Y but high affinity for full length neuropeptide Y and peptide YY (Wahlestedt et al., 1986). More recently, it was discovered that replacing amino acids 31 and 34 of neuropeptide Y (Fuhlendorff et al., 1990) or peptide YY (Dumont et al., 1994) with the leucine and proline, respectively, of pancreatic polypeptide

* Corresponding author. Tel.: (1-317) 276-1810; Fax: (1-317) 276-5546; e-mail: Gehlert_Donald_R@Lilly.com

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created a highly selective peptide for the neuropeptide Y Y_1 receptor over the neuropeptide Y Y_2 receptor. Further studies revealed that only the Pro^{34} substitution was necessary for neuropeptide Y Y_1 selectivity (Potter et al., 1991). The substituted neuropeptide Y and peptide YY peptides have been widely used to define the role of neuropeptide Y Y_1 in various central and peripheral functions. [Leu^{31},Pro^{34}]Neuropeptide Y (Larsen, 1993) and [Leu^{31},Pro^{34}]peptide YY (Dumont et al., 1995) have been radioiodinated and the binding characterized in rat brain membrane homogenate preparations. Both radioligands had high affinity and pharmacology consistent with neuropeptide Y Y_1 receptor binding. Pro^{34}-substituted neuropeptide Y and peptide YY analogs also have relatively high affinity for human pancreatic polypeptide 1 receptors (Gehlert et al., 1996). Therefore, we characterized the binding of

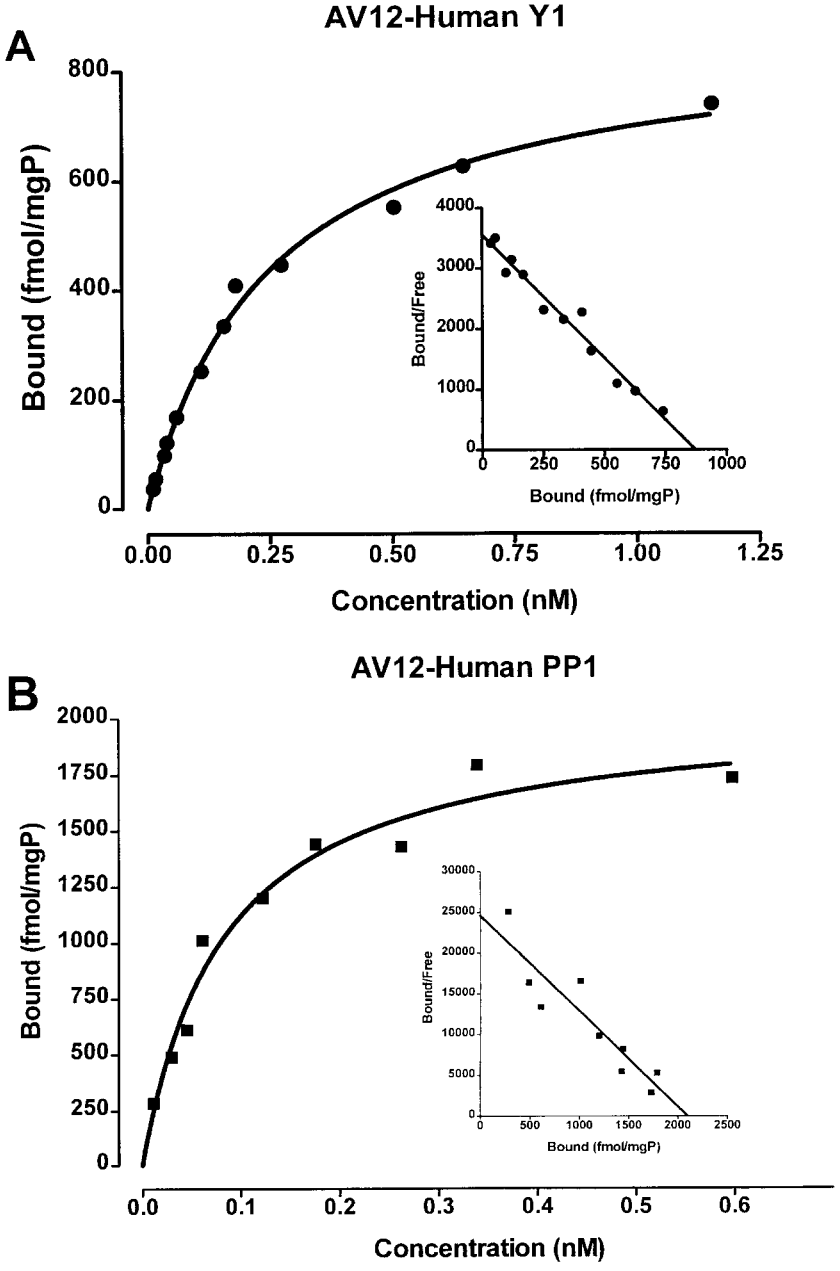

Fig. 1. A: Representative saturation analysis and Scatchard plot (inset) using [^{125}I][Leu^{31},Pro^{34}]peptide YY binding to AV12-human neuropeptide Y Y_1 (AV12-human Y1) cell membranes as described in Section Section 2. The K_d value was 240 ± 10 pM with a B_{max} of 820 ± 10 fmol/mg protein (mean ± S.E.M., $n = 4$). Nonspecific binding was determined by including 1 μM human neuropeptide Y in the incubation buffer. B: Representative saturation analysis and Scatchard plot (inset) using [^{125}I][Leu^{31},Pro^{34}]peptide YY binding to AV12-human pancreatic polypeptide 1 (AV12-human PP1) cell membranes. The K_d value was 120 ± 10 pM with a B_{max} of 2050 ± 130 fmol/mg protein (mean ± S.E.M., $n = 8$). Nonspecific binding was determined by including 100 nM human pancreatic polypeptide in the incubation buffer.

[^{125}I][Leu^{31},Pro^{34}]peptide YY to cell lines transfected with the human neuropeptide Y Y_1 and human pancreatic polypeptide 1 receptors.

2. Materials and methods

2.1. Expression of human pancreatic polypeptide 1 and human neuropeptide Y Y_1 in AV12 cells

A cDNA encoding the human neuropeptide Y Y_1 receptor was cloned from a human fetal cDNA library (Stratagene) and subcloned into the expression vector pHD. The constructs were transfected into AV12 cells using the calcium phosphate procedure. The cells were maintained under 95%/5% O_2/CO_2 at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine and 100 μg/ml steptomycin. Stably transfected cells were selected with 500 μg/ml G418 and tested for their ability to bind [^{125}I]peptide YY (Dupont-NEN, Boston, MA, USA). The human pancreatic polypeptide 1 cDNA (Lundell et al., 1995) was subcloned into pHD and transfected into the AV12 cell line using Lipofectin (Gibco-BRL, Gaithersburg, MD, USA). The cells were initially selected with 1 μM methotrexate and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin and 200 μg/ml hygromycin.

2.2. Receptor binding assays

Cells were washed once with phosphate buffered saline (PBS) and scraped into 10 ml PBS. The detached cells were pelleted by centrifugation (800 × *g*, 5 min) and stored at −80°C until used. The assay was conducted in a final volume of 200 μl containing 100 pM [^{125}I][Leu^{31},Pro^{34}]peptide YY (New England Nuclear, Boston, MA, USA) and 3–10 μg protein of AV12-human neuropeptide Y Y_1 membranes or 0.4–0.5 μg protein of AV12-human pancreatic polypeptide 1 membranes. The incubation buffer contained 25 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 2 g/l bacitracin (pH 7.4). Experiments were incubated at room temperature for 2 h. Nonspecific binding was defined as the radioactivity remaining bound to the membranes after incubation with the radioligand and 1 μM human neuropeptide Y (Peninsula, Belmont, CA, USA) for the AV12-human neuropeptide Y Y_1 cell line or 100 nM human pancreatic polypeptide (Bachem, King of Prussia, PA, USA) for the AV12-human pancreatic polypeptide 1 cell line. For saturation studies, cell membranes were incubated with various concentrations of [^{125}I][Leu^{31},Pro^{34}]peptide YY. Competition studies used various concentrations of the indicated peptides and 100 pM [^{125}I][Leu^{31},Pro^{34}]peptide YY in the incubation buffer. Incubations were terminated by rapid filtration (Tomtec cell harvester, Orange, CT, USA) through Wallac Printed Filtermat A glass fiber filters (Wallac, Gaithersburg, MD, USA) presoaked in 0.3% polyethyleneimine (Sigma, St. Louis, MO, USA). The filters were dried in a heated oven. Radioactivity retained on the filters was counted using a solid scintillation system and a Wallac Betaplate counter (Wallac). The results were analyzed using Microsoft Excel (Cheng-Prusoff equation) and Prism (GraphPad, San Diego, CA, USA).

3. Results

The transfection procedures resulted in cell lines exhibiting high levels of specific binding for [^{125}I]peptide YY. One clonal cell line was selected for each receptor for further study. The human neuropeptide Y Y_1-transfected cell line AV12-human neuropeptide Y Y_1 exhibited high

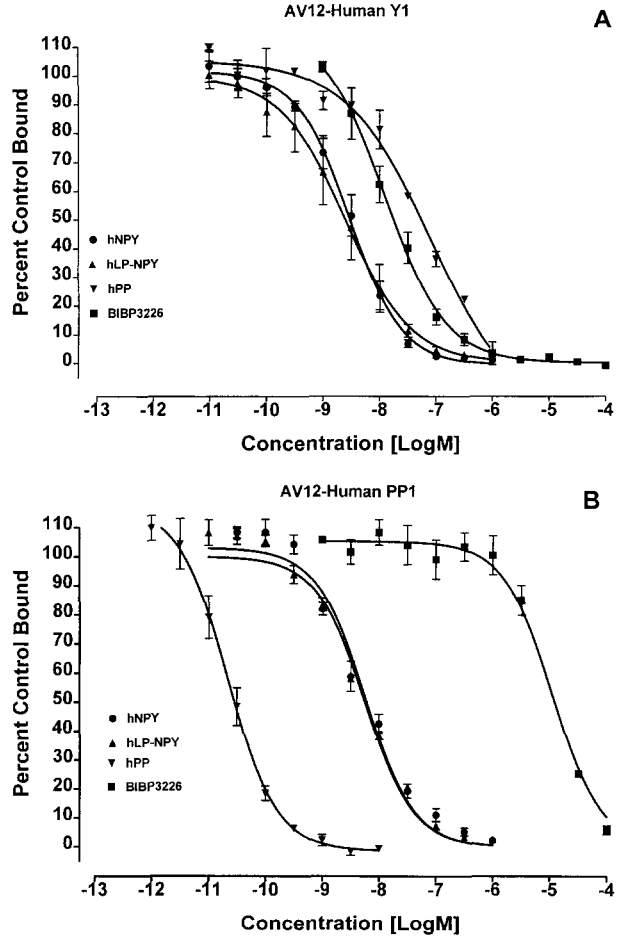

Fig. 2. A: Displacement curves for [^{125}I][Leu^{31},Pro^{34}]peptide YY using AV12-human neuropeptide Y Y_1 (AV12-human Y1) cell membrane homogenates ($n = 2$–4 experiments run in duplicate). B: Displacement curves for [^{125}I][Leu^{31},Pro^{34}]peptide YY using AV12-human pancreatic polypeptide 1 (AV12-human PP1) cell membrane homogenates ($n = 2$–4 experiments run in duplicate).

affinity specific binding of [^{125}I][Leu^{31},Pro^{34}]peptide YY (Fig. 1A). The percentage of specific binding at a concentration of 100 pM averaged 80.1%. The binding was characterized by a K_d of 240 pM and a B_{max} of 820 fmol/mg protein. The AV12-human pancreatic polypeptide 1 cell line also exhibited high affinity binding of [^{125}I][Leu^{31},Pro^{34}]peptide YY with a K_d of 120 pM and a B_{max} of 2050 fmol/mg protein (Fig. 1B). The average specific binding for the AV12-human pancreatic polypeptide 1 cell line at a concentration of 100 pM was 84.5%. To further characterize the binding of [^{125}I][Leu^{31},Pro^{34}]peptide YY to these cell lines, the ability of a variety of peptide fragments, analogs and orthologs to inhibit binding was tested. These data are summarized in Table 1 and Figs. 2 and 3. In the AV12-human neuropeptide Y Y_1 cell line, salmon pancreatic polypeptide, human peptide YY and human [Leu^{31},Pro^{34}]peptide YY were the most potent inhibitors of binding with K_i values of 0.03 nM, 0.16 nM and 0.20 nM respectively. Several pancreatic polypeptide orthologs were tested. Avian pancreatic polypeptide, bovine pancreatic polypeptide, human pancreatic polypeptide and rat pancreatic polypeptide were less potent than salmon pancreatic polypeptide (K_i = 0.03 nM) with K_i values of 18.2 nM, 14.3 nM, 4.2 nM and 26.8 nM, respectively (Fig. 2A and Fig. 3A). The 3–36 fragment of peptide YY was substantially less potent than peptide YY with a K_i of 37 nM. Human neuropeptide Y was less potent than human peptide YY with a K_i of 1.2 nM. While the 3–36 fragment of neuropeptide Y also showed significantly lower affinity with a K_i of 160 nM, neuropeptide Y-(2–36) retained most of the affinity of the native peptide with a K_i of 4.4 nM. Porcine [Leu^{31},Pro^{34}]neuropeptide Y also had affinity similar to human neuropeptide Y with a K_i of 1.3 nM. The pharmacology of neuropeptide Y and peptide YY analogs at the AV12-human pancreatic polypeptide 1 cell line was similar to the neuropeptide Y Y_1 cell line for the Pro^{34}-subtituted peptides but varied for the C-teminal fragments (Table 1, Fig. 2). The pancreatic polypeptide orthologs were more potent at the human pancreatic polypeptide 1 receptor (Table 1, Fig. 2B and Fig. 3B). The most potent ortholog was bovine pancreatic polypeptide with a K_i of

Table 1
Pharmacology of [^{125}I][Leu^{31},Pro^{34}]peptide YY binding to cell lines expressing the human neuropeptide Y Y_1 and human pancreatic polypeptide 1 receptor

Peptide	AV12-hNPY Y_1 K_i (nM)	AV12-hPP1 K_i (nM)
Neuropeptide Y		
Human	1.2 ± 0.4	2.8 ± 0.5
Porcine fragment 2–36	4.4 ± 1.5	11.4 ± 0.6
Porcine fragment 3–36	160 ± 50	55 ± 5
Porcine Leu^{31},Pro^{34}	1.3 ± 0.7	1.8 ± 0.7
Peptide YY		
Human	0.16 ± 0.02	0.31 ± 0.03
Human fragment 3–36	37 ± 15	8.6 ± 2.0
Human Leu^{31},Pro^{34}	0.20 ± 0.03	0.38 ± 0.06
Pancratic polypeptide		
Avian	18.2 ± 4.7	0.18 ± 0.07
Bovine	14.3 ± 3.7	0.009 ± 0.002
Human	4.2 ± 0.3	0.02 ± 0.002
Rat	26.8 ± 0.4	0.02 ± 0.005
Salmon	0.03 ± 0.004	0.25 ± 0.03
Antagonist		
BIBP3226	3.4 ± 0.4	4250 ± 400

K_i values represent the average (± S.E.M.) of 3 or 4 experiments run in duplicate at a radioligand concentration of 100 pM as described in Section Section 2. Nonspecific binding was determined by including 1 μM human neuropeptide Y with AV12-human neuropeptide Y Y_1 (AV12-hNPY Y_1) cell membranes or 100 nM human pancreatic polypeptide with AV12-human pancreatic polypeptide 1 (AV12-hPP1) cell membranes in the incubation buffer.

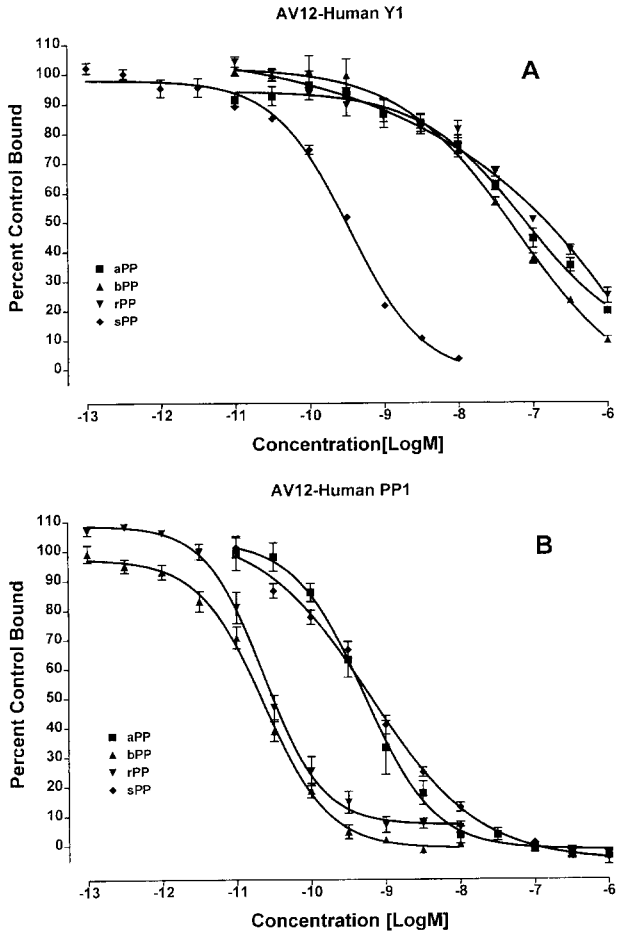

Fig. 3. A: Displacement curves of pancreatic polypeptide orthologs for [^{125}I][Leu^{31},Pro^{34}]peptide YY using AV12-human neuropeptide Y Y_1 (AV12-human Y1) cell membrane homogenates (n = 2–4 experiments run in duplicate). B: Displacement curves of pancreatic polypeptide orthologs for [^{125}I][Leu^{31},Pro^{34}]peptide YY using AV12-human pancreatic polypeptide 1 (AV12-human PP1) cell membrane homogenates (n = 2–4 experiments run in duplicate).

0.009 nM representing greater than 1500-fold selectivity for the human pancreatic polypeptide 1 receptor. Avian pancreatic polypeptide, human pancreatic polypeptide and rat pancreatic polypeptide had similar high affinities for human pancreatic polypeptide 1 with 100-fold, 250-fold and 500-fold selectivity. Interestingly, salmon pancreatic polypeptide was the most potent inhibitor of [^{125}I][Leu^{31},Pro^{34}]peptide YY binding in the human neuropeptide Y Y_1 cell line and was 10-fold more selective for the neuropeptide Y Y_1 receptor compared to the human pancreatic polypeptide 1 receptor. The neuropeptide Y Y_1 selective antagonist, BIBP3226, was over 1000-fold more selective for the neuropeptide Y Y_1 receptor versus the pancreatic polypeptide 1 (Fig. 2). Human peptide YY and human neuropeptide Y exhibited similar affinities for the two receptor subtypes and the C-terminal fragments of porcine neuropeptide Y and human peptide YY had reduced potency for both receptors. [Leu^{31},Pro^{34}]Peptide YY and [Leu^{31},Pro^{34}]neuropeptide Y had similar affinities for the human pancreatic polypeptide 1 receptor when compared to neuropeptide Y Y_1.

4. Discussion

Like the neuropeptide Y Y_1 receptor, the recently discovered human pancreatic polypeptide 1 receptor exhibits reasonably high affinity for all the known peptides of the pancreatic polypeptide-fold peptide family (Lundell et al., 1995). Unlike the human neuropeptide Y Y_1, the human pancreatic polypeptide 1 receptor exhibits very high affinity for most pancreatic polypeptide orthologs while having approximately 10-fold lower affinity for human peptide YY and 100-fold lower affinity for human neuropeptide Y. This differs from the rank order of potency for the neuropeptide Y Y_1 receptor where human peptide YY and human neuropeptide Y are the most potent while the pancreatic polypeptide orthologs, except salmon pancreatic polypeptide, are somewhat less potent. Pro^{34}-substituted analogs of porcine neuropeptide Y and human peptide YY have been considered to be selective ligands for the neuropeptide Y Y_1 receptor and have been used extensively to explore neuropeptide Y Y_1-mediated responses in vitro and in vivo. The substitution at position 34 was introduced to replace the Gln^{34} found in porcine neuropeptide Y with the Pro^{34} found in human pancreatic polypeptide (Fuhlendorff et al., 1990). While this change produced peptide ligands that were selective for neuropeptide Y Y_1 over neuropeptide Y Y_2, it now appears that this exchange also preserves high affinity for the human pancreatic polypeptide 1 receptor in vitro. In the present study, we found [^{125}I][Leu^{31},Pro^{34}]peptide YY bound to the human pancreatic polypeptide 1 with higher affinity when compared to human neuropeptide Y Y_1. Thus, at the human receptors, it appears that Pro^{34}-substituted peptides bind with high affinity to both human neuropeptide Y Y_1 and human pancreatic polypeptide 1 while having much lower affinity at the human neuropeptide Y Y_2 receptor. The pharmacology of [^{125}I][Leu^{31},Pro^{34}]peptide YY binding to the two receptors was similar with the major differences being the very high affinity of the pancreatic polypeptide orthologs for the human pancreatic polypeptide 1 receptor and the high affinity of BIBP3226 for the neuropeptide Y Y_1 receptor. Bovine pancreatic polypeptide was the most selective pancreatic polypeptide ortholog with a 1500-fold preference for the human pancreatic polypeptide 1 receptor. The nonpeptide neuropeptide Y Y_1 antagonist, BIBP3226, exhibited over a 1000-fold selectivity for the neuropeptide Y Y_1 receptor.

While the human pancreatic polypeptide 1 receptor appears to bind all of the pancreatic polypeptide-fold peptides with relatively high affinity, the recently cloned rat ortholog does not appear to share that property (Lundell et al., 1996). This receptor has relatively poor conservation at the amino-acid level with only 75% identity to the human sequence. While rat pancreatic polypeptide 1 also binds pancreatic polypeptide orthologs with very high affinity, this receptor exhibits relatively poor affinity for rat neuropeptide Y and rat peptide YY. The K_i values for bovine pancreatic polypeptide, human pancreatic polypeptide and rat pancreatic polypeptide are 0.0181 nM, 0.0135 nM and 0.0193, respectively, compared to 1.17 nM and 3.53 nM for rat neuropeptide Y and rat peptide YY (Gehlert et al., in preparation). This suggests the rat pancreatic polypeptide 1 receptor has a pharmacology that is more similar to the pharmacology traditionally assigned to the pancreatic polypeptide receptor as described in PC-12 cells (Schwartz et al., 1987). Interestingly, this substitution of Pro^{34} into both human peptide YY and porcine neuropeptide Y dramatically improves the affinity of these two peptides for rat pancreatic polypeptide 1. This appears to differ from human pancreatic polypeptide 1 where the Pro^{34} substitution results in peptides with similar affinity when compared to the native peptide. The rat pancreatic polypeptide 1 receptor also binds [^{125}I][Leu^{31},Pro^{34}]peptide YY with high affinity (Gehlert et al., in preparation). Thus, Pro^{34} peptides bind to the rat pancreatic polypeptide 1 receptor and are not completely selective for the neuropeptide Y Y_1 receptor in this species either. In addition, these data are consistent with differences in how the pancreatic polypeptide 1 receptor recognizes key amino acids in the pancreatic polypeptide sequence when compared to human neuropeptide Y Y_1 and human neuropeptide Y Y_2. Further work is in progress to study the pancreatic polypeptide binding epitope in human pancreatic polypeptide 1 and rat pancreatic polypeptide 1.

In summary, [^{125}I][Leu^{31},Pro^{34}]peptide YY binds with high affinity to human neuropeptide Y Y_1 and human pancreatic polypeptide 1 receptors. In addition, Pro^{34}-substituted porcine neuropeptide Y and human peptide YY analogs are high affinity ligands for both receptor subtypes. Data obtained with this radioligand and with Pro^{34}-substituted analogs should be interpreted with this in mind.

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