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Cloning and functional expression of cDNAs encoding human and rat pancreatic polypeptide receptors

(neuropeptide Y/peptide YY/G-protein-coupled receptor)

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ABSTRACT **PCR was used to isolate nucleotide sequences that may encode novel members of the neuropeptide Y receptor family. By use of a PCR product as a hybridization probe, a full-length human cDNA was isolated that encodes a 375-aa protein with a predicted membrane topology identifying it as a member of the G-protein-coupled receptor superfamily. After stable transfection of the cDNA into human embryonic kidney 293 cells, the receptor exhibited high affinity (K_d = 2.8 nM) for ^{125}I-labeled human pancreatic polypeptide (PP). Competition binding studies in whole cells indicated the following rank order of potency: human PP = bovine PP ≥ human [Pro^{34}]peptide YY > rat PP > human peptide YY = human neuropeptide Y. Northern blot analysis revealed that human PP receptor mRNA is most abundantly expressed in skeletal muscle and, to a lesser extent, in lung and brain tissue. A rat cDNA clone encoding a high-affinity PP receptor that is 74% identical to the human PP receptor at the amino acid level was also isolated. These receptor clones will be useful in elucidating the functional role of PP and designing selective PP receptor agonists and antagonists.**

Pancreatic polypeptide (PP), peptide YY (PYY), and neuropeptide Y (NPY) are C-terminal amidated, 36-aa peptides that constitute the mammalian NPY family (1). PP is produced predominantly by pancreatic islet cells and regulates pancreatic exocrine secretion through a mechanism that is not fully understood but may involve inhibition of vagal input to the pancreas (2, 3). PYY-like immunoreactivity is found in endocrine cells of the lower gastrointestinal tract, and the peptide has been shown to have a variety of effects on gastrointestinal function (4, 5). NPY, which is the most highly conserved of the family members between species (6), is extensively distributed throughout both the central and the peripheral nervous systems (7, 8). NPY is colocalized with norepinephrine in postganglionic sympathetic nerves (4) and is a potent vasopressor agent (9). The observations of persistent vasoconstriction after α-adrenergic receptor blockade (10, 11) and elevated levels of NPY in hypertensive patients (10, 12) suggest a possible role for NPY in the pathophysiology of hypertension. In the central nervous system, NPY immunoreactivity and mRNA are prevalent in various hypothalamic nuclei, the cerebral cortex, and the septal-hippocampal system and striatum (5). Important effects of centrally administered NPY include hyperphagia (13), anxiolysis (14), and regulation of pituitary hormone release (15).

Receptors for NPY/PYY have been delineated on the basis of pharmacological selectivity exhibited by certain tissues and cell lines. The Y1 receptor exhibits high affinity for NPY, PYY, and C-terminal-substituted variants, including [Leu^{31},Pro^{34}]NPY, but low affinity for long C-terminal fragments such as NPY-13–36 (16, 17). This receptor, which is a member of the G-protein-coupled receptor superfamily, has been cloned (18), and its pharmacological properties in transfected cells are identical to those of Y1 receptors expressed in native tissues (16, 19). In contrast to Y1 receptors, Y2 receptors have high affinity for C-terminal fragments but low affinity for C-terminal-substituted analogs of NPY and PYY (16, 17). Cloning of a human cDNA encoding the Y2 receptor has recently been reported (20). The least studied of the NPY receptors is the Y3 receptor, which, unlike Y1 and Y2 receptors, recognizes NPY but not PYY and has high affinity for NPY C-terminal fragments as well as C-terminal-substituted analogs (16).

PP has negligible affinity for Y1, Y2, and Y3 receptors, but high-affinity PP receptors that do not recognize NPY or PYY have been described on rat pheochromocytoma (PC12) cells and in rat brainstem (3, 21). The present study describes the cloning and functional expression of human and rat PP receptors from brain cDNA libraries. Given that PP has profound behavioral effects after central administration (22) and that the sites of action and mechanisms underlying both its central and peripheral effects are poorly understood, the cloning of these receptors represents an important step toward understanding the biological properties of PP.

MATERIALS AND METHODS

PCR. Degenerate oligonucleotide primers corresponding to conserved amino acids within the sixth and seventh transmembrane regions of NPY Y1 and neurokinin receptors were synthesized. The forward primer pool sequence was GTG-GT(A/C)GC(A/C)CT(T/C)GC(C/G/A)GTCTGCTGGCT, encoding the amino acid sequence VVALAVCW. The reverse primer pool sequence was (T/G)ATGGGGTTGAC(A/G)CA(A/G)GTGGA(T/G)ATCAT, corresponding to the amino acid sequence MISTCVNPI. PCR was first applied to genomic DNA of the pufferfish *Fugu rubripes*, and the products were sequenced. Cloning of the full-length *F. rubripes* sequence will be described elsewhere. The same primers were applied to 200 ng of human genomic DNA using a Perkin–Elmer GeneAmp PCR System 9600. Resulting PCR fragments of expected size (≈150 bp) were isolated, subcloned, and sequenced. One clone, designated as human 1b, contained a 99-bp insert between the primer regions and represented a novel sequence with significant homology to the correspond-

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Abbreviations: PP, pancreatic polypeptide; NPY, neuropeptide Y; PYY, peptide YY; HEK, human embryonic kidney; ^{125}I-hPP, ^{125}I-labeled human PP; ^{125}I-rPP, ^{125}I-labeled rat PP.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U42387, human pancreatic polypeptide receptor; U42388, rat pancreatic polypeptide receptor; and U42389, human neuropeptide Y (Y2) receptor].

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ing region of human NPY Y1 receptor and the *F. rubripes* receptor fragment previously isolated.

cDNA Cloning of Human and Rat PP Receptors. The aforementioned 99-bp fragment was radiolabeled with [α-^{32}P]dCTP [Amersham; specific activity = 3000 Ci/mmol (1 Ci = 37 GBq)] and used as a hybridization probe to screen $\approx 1.2 \times 10^6$ clones from a human fetal brain cDNA library (Clontech). Hybridization was performed overnight at 60°C in 6× standard saline citrate (SSC)/1× Denhardt's solution/ 0.5% SDS/50 μg/ml of single-stranded salmon sperm DNA. Final wash conditions were 0.2× SSC/0.5% SDS at 60°C. A single hybridization-positive plaque was isolated and sequenced. The 5′ end of the full-length sequence was obtained by performing nested PCR on a lysate of a human brain cDNA library employing specific antisense primers.

Oligonucleotide primers specific to different regions of the human PP receptor cDNA were synthesized and used as PCR primers at moderate stringency to amplify corresponding regions of the rat sequence from a lysate of a rat whole brain cDNA library. The resulting PCR fragments were subcloned and used as hybridization probes to isolate full-length cDNA encoding the rat PP receptor.

Functional Expression of Human and Rat PP Receptors in Human Embryonic Kidney (HEK) 293 Cells. Full-length cDNA for the human PP receptor was subcloned into a cytomegalovirus promoter-based eukaryotic expression vector in both sense and antisense orientations. HEK 293 cells were transiently transfected as described (23). Transfectants were maintained in DMEM containing 10% fetal bovine serum for 48 h before harvesting. Cell membrane suspensions were tested for their ability to bind ^{125}I-labeled human pancreatic polypeptide (^{125}I-hPP) as described below. Cells transfected with antisense expression constructs served as negative controls.

HEK 293 cells stably expressing the human PP receptor were generated by cotransfection of the sense expression construct with psvNeo$^+$ plasmid. Individual G418-resistant colonies were selected and amplified. Positive stable cell lines were identified by specific binding of ^{125}I-hPP. One colony, designated as h2d, was selected for analysis.

Similarly, an expression vector containing full-length rat PP receptor cDNA was constructed and transiently transfected into 293 cells. Transfectants were tested for their ability to bind ^{125}I-labeled rat pancreatic polypeptide (^{125}I-rPP). Stable HEK 293 cell lines expressing the rat PP receptor were also generated as described above. One colony, designated as r44, was selected for further study.

Radioligand Binding. Synaptic membrane-enriched homogenates (24) prepared from transiently transfected HEK 293 cells were washed twice in 50 mM Hepes (pH 7.4) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% BSA, 0.1 mg/ml of soybean trypsin inhibitor (STI), 0.1 mg/ml of 4-(2-aminoethyl)benzenesulfonyl fluoride HCl (AEBSF), and 0.5 mg/ml of bacitracin (buffer A). The binding reaction was initiated by the addition of membranes from 0.5×10^6 cells to tubes containing 20–30 pM ^{125}I-hPP (Amersham; specific activity = 2200 Ci/mmol) or ^{125}I-rPP (New England Nuclear; specific activity = 2200 Ci/mmol) and was terminated after a 90-min incubation at room temperature by filtration over Whatman GF/C filters previously soaked in 0.5% polyethyleneimine. Specific binding (typically >90%) was defined as the percentage of total binding displaceable by 0.3 μM unlabeled human PP or rat PP. Membrane-bound radioactivity was quantitated using a γ counter. All assays were performed in triplicate.

Saturation analysis and competition studies in stably transfected HEK 293 cells were performed using intact cells plated at a density of $1\text{-}2 \times 10^5$ cells per well in fibronectin-coated plates. After 48 h, the growth medium was removed and replaced with MEM with Earle's salts containing 25 mM Hepes (pH 7.4), 0.1% BSA, 0.1 mg/ml of STI, 0.1 mg/ml of AEBSF, and 0.5 mg/ml of bacitracin (buffer B). For saturation analysis, the binding reaction was initiated by the addition of ^{125}I-hPP to yield final concentrations ranging between 0.1 and 10 nM. Specific binding at each radioligand concentration was defined in the presence of 0.3 μM unlabeled human PP. The reaction was conducted for 120 min at room temperature and terminated by removal of buffer B, followed by rinsing in ice-cold MEM containing 25 mM Hepes (pH 7.4) and solubilization in 1.0 M NaOH. Cell-associated radioactivity was quantitated using a γ counter.

Competition studies were performed as described above, except that the reaction mixture contained 20-30 pM ^{125}I-hPP or ^{125}I-rPP and various concentrations of unlabeled test peptides.

Binding data were analyzed using an iterative curve-fitting program (GraphPad, San Diego).

cAMP Assay. cAMP accumulation was assayed in HEK 293 cells stably transfected with human PP receptor cDNA as described (25). cAMP was quantitated using a radioimmunoassay kit (Amersham).

Northern Blot Analysis. Northern blots containing RNA from multiple human peripheral tissues and brain regions were purchased from Clontech. Each lane contained 2 μg of poly(A)$^+$ RNA. The blots were hybridized with a ^{32}P-radiolabeled probe representing full-length human PP receptor cDNA. Conditions for hybridization were 0.25 M Na_2HPO_4/0.25 M NaH_2PO_4/5 mM EDTA/1% BSA/7% SDS at 60°C overnight. Final wash conditions were 0.1× SSC/0.1% SDS at 60°C. The integrity of the mRNA was confirmed by the presence of intact β-actin transcript.

RESULTS AND DISCUSSION

Isolation of Novel Human and Rat cDNAs Encoding NPY Receptor Family Members. PCR employing oligonucleotide pools corresponding to regions of amino acid sequence homology between NPY Y1 and neurokinin receptors served to isolate a novel 99-bp DNA fragment from human genomic DNA. This fragment had the potential to encode 33 amino acids exhibiting 54% identity to the corresponding region within the human NPY Y1 receptor. This DNA fragment was subsequently used as a hybridization probe to isolate two overlapping cDNA clones from human fetal brain and human adult brain cDNA libraries from which the full-length cDNA was constructed. The nucleotide and deduced protein sequences are shown in Fig. 1. The initiation codon begins at nucleotide 86 and is preceded by several in-frame stop codons. The full-length receptor cDNA has an open reading frame encoding a 375-aa protein with a predicted molecular mass of 42 kDa. Hydropathicity analysis (26) revealed the presence of seven hydrophobic segments, indicative of the transmembrane topology that defines the G-protein-coupled receptor superfamily.

Oligonucleotide primers specific to the human receptor cDNA were synthesized and used to amplify the corresponding region of the rat receptor from a lysate of a rat whole brain cDNA library. The PCR product was then used as a hybridization probe to isolate the full-length cDNA from the same rat brain library. The open reading frame of the full-length rat receptor cDNA also encodes a 375-aa protein.

Pharmacological and Functional Properties of Expressed Receptors. Complementary DNA encoding the receptors was subcloned into a eukaryotic expression vector and transiently transfected into HEK 293 cells to determine if the expressed receptors were capable of binding NPY-related ligands. Radioligand binding studies in membranes prepared from transiently transfected HEK 293 cells indicated that the human receptor has high affinity for ^{125}I-hPP (data not shown). Further characterization of the receptor was performed in stably transfected HEK 293 cells (clone h2d). Nonlinear regression analysis of saturation binding performed in whole

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TTAATAATGACTAGAGAATCTGAGAGGCGTCATCCCTCAAGTGTATCACTTAGTTCAAGA   60
GTCCTGGAATCTTTTCACATCCACTATGAACACCTCTCACCTCCTGGCCTTGCTGCTCCC  120
                    M  N  T  S  H  L  L  A  L  L  L  P
AAAATCTCCACAAGGTGAAAACAGAAGCAAACCCCTGGGCACCCCATACAACTTCTCTGA  180
 K  S  P  Q  G  E  N  R  S  K  P  L  G  T  P  Y  N  F  S  E
ACATTGCCAGGATTCCGTGGACGTGATGGTCTTCATCGTCACTTCCTACAGCATTGAGAC  240
                                          I
 H  C  Q  D  S  V  D  V  M  V  F  I  V  T  S  Y  S  I  E  T
TGTCGTGGGGGTCCTGGGTAACCTCTGCCTGATGTGTGTGACTGTGAGGCAGAAGGAGAA  300
 V  V  G  V  L  G  N  L  C  L  M  C  V  T  V  R  Q  K  E  K
AGCCAACGTGACCAACCTGCTTATCGCCAACCTGGCCTTCTCTGACTTCCTCATGTGCCT  360
                                      II
 A  N  V  T  N  L  L  I  A  N  L  A  F  S  D  F  L  M  C  L
CCTCTGCCAGCCGCTGACCTCCGTCTACACCATCATGGACTACTGGATCTTTGGAGAGAC  420
 L  C  Q  P  L  T  S  V  Y  T  I  M  D  Y  W  I  F  G  E  T
CCTCTGCAAGATGTCGGCCTTCATCCAGTGCATGTCGGTGACGGTCTCCATCCTCTCGCT  480
                                          III
 L  C  K  M  S  A  F  I  Q  C  M  S  V  T  V  S  I  L  S  L
CGTCCTCGTGGCCCTGGAGAGGCATCAGCTCATCATCAACCCAACAGGCTGGAAGCCCAG  540
 V  L  V  A  L  E  R  H  Q  L  I  I  N  P  T  G  W  K  P  S
CATCTCACAGGCCTACCTGGGGATTGTGCTCATCTGGGTCATTGCCTGTGTCCTCTCCCT  600
                                      IV
 I  S  Q  A  Y  L  G  I  V  L  I  W  V  I  A  C  V  L  S  L
GCCCTTCCTGGCCAACAGCATCCTGGAGAATGTCTTCCACAAGAACCACTCCAAGGCTCT  660
 P  F  L  A  N  S  I  L  E  N  V  F  H  K  N  H  S  K  A  L
GGAGTTCCTGGCGGATAAGGTGGTCTGTACCGAGTCCTGGCCACTGGCTCACCACCGCAC  720
 E  F  L  A  D  K  V  V  C  T  E  S  W  P  L  A  H  H  R  T
CATCTACACCACCTTCCTGCTCCTCTTCCAGTACTGCCTCCCACTGGGCTTCATCTTGGT  780
                                  V
 I  Y  T  T  F  L  L  L  F  Q  Y  C  L  P  L  G  F  I  L  V
CTGTTATGCACGCATCTACCGGCGCCTGCAGAGGCAGGGGCGCGTGTTTCACAAGGGCAC  840
 C  Y  A  R  I  Y  R  R  L  Q  R  Q  G  R  V  F  H  K  G  T
CTACAGCTTGCGAGCTGGGCACATGAAGCAGGTCAATGTGGTGCTGGTGGTGATGGTGGT  900
                                          VI
 Y  S  L  R  A  G  H  M  K  Q  V  N  V  V  L  V  V  M  V  V
GGCCTTTGCCGTGCTCTGGCTGCCTCTGCATGTGTTCAACAGCCTGGAAGACTGGCACCA  960
 A  F  A  V  L  W  L  P  L  H  V  F  N  S  L  E  D  W  H  H
TGAGGCCATCCCCATCTGCCATGGGAACCTCATCTTCTTAGTGTGCCACTTGCTTGCCAT 1020
                                      VII
 E  A  I  P  I  C  H  G  N  L  I  F  L  V  C  H  L  L  A  M
GGCCTCCACCTGTGTCAACCCATTCATCTATGGCTTTCTCAACACCAACTTCAAGAAGGA 1080
 A  S  T  C  V  N  P  F  I  Y  G  F  L  N  T  N  F  K  K  E
GATCAAGGCCCTGGTGCTGACTTGCCAGCAGAGCGCCCCCCTGGAGGAGTCAGAGCATCT 1140
 I  K  A  L  V  L  T  C  Q  Q  S  A  P  L  E  E  S  E  H  L
GCCCCTGTCCACAGTACATACGGAAGTCTCCAAAGGGTCCCTGAGGCTAAGTGGCAGGTC 1200
 P  L  S  T  V  H  T  E  V  S  K  G  S  L  R  L  S  G  R  S
CAATCCCATTTAACCAGGTCTAGGTCTTCTCCCTGCCATGTCCCTTGCCAGGCTCTTCCA 1260
 N  P  I  *
CTTAGCTAAGTGGGCACACTGCAAGCTGGGGTGGCACCCCAGCATTCCTGGCTTTCTGGG 1320
GTCCAGATAGGCTGGCAAGAGCTGTTTTTGCATCCATTTGCATCGTGAAGACTGGCATTT 1380
TGATACTTCAGCTGTTTGTTCCTGGGAGAATTCTGAGCACAGATTCCAGAGGTCACAGTA 1440
AGCCTTGCAGCTTGAGCTGAAAGATGCCAGAGCCGGAGATGTCTGCTGGCAGCAGGCAGG 1500
GTTCATTCTGGTGACACAGCAACAGATGCCTGGCCTGGGAACCCAGGGATTTCACCTCCA 1560
CCAGTGAGACCACGGGGCCACTGTGGGGTGAGGGAAGGAGCGCTTGGAGTCAGAGCTCTA 1620
GAGTCGACCTGCAGGCATGCAAGCTTGGCACTGAAGCCGAATTCCAGCACACT        1673
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FIG. 1. cDNA and deduced amino acid sequences of the human PP receptor. Positions of the putative transmembrane segments I–VII of the receptor are indicated by the boxes. Potential N-glycosylation sites are underlined and conserved cysteine residues are circled. The amino acid sequence coded by the initial PCR product is highlighted.

cells revealed a dissociation constant (K_d) of 2.8 ± 0.6 nM (n = 3) for ^{125}I-hPP (Fig. 2). The rank order of potency as determined in competition studies was human PP = bovine PP ≥ human [Pro^{34}]PYY > rat PP > human PYY = human NPY (Table 1). Replacement of glutamine by proline in position 34 of human PYY, a modification that has been shown to impart Y1 receptor selectivity (27), yielded a compound with a potency nearly equal to that of human PP at the human receptor. This finding demonstrates the importance of the C-terminal portion of the peptide in determining agonist potency and suggests that caution must be taken in assigning the functional effects of C-terminal-substituted NPY and PYY analogs solely to Y1 receptor activation. However, portions of the peptide other than the C terminus must be important for binding to the human PP receptor because rat PP, whose C-terminal pentapeptide sequence is identical to both human PP and human [Pro^{34}]PYY, is nonetheless much less potent than these compounds in inhibiting ^{125}I-hPP binding.

In common with the human receptor, the rat PP receptor (clone r44) exhibited a marked preference for PP over NPY

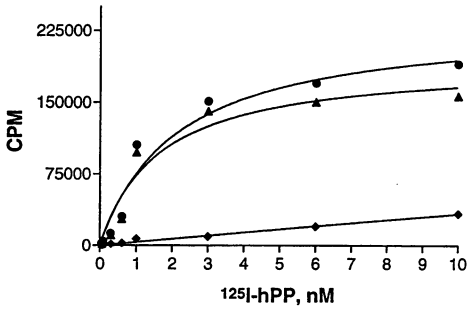

FIG. 2. Saturation binding isotherm of ^{125}I-hPP binding to HEK 293 cells stably transfected with human PP receptor cDNA. The data shown are from a representative experiment, and each point is the mean of triplicate determinations. The experiment was repeated two additional times with similar results. Untransfected HEK 293 cells showed no specific ^{125}I-hPP binding. ●, Total; ▲, specific; ◆, nonspecific.

and PYY and had substantially greater affinity for human [Pro^{34}]PYY than for human PYY (Table 1). However, in contrast to the findings with the human receptor, rat and human PP are nearly equipotent inhibitors of ^{125}I-rPP binding to the rat receptor (Table 1), suggesting that the structural requirements for agonist binding to the rat PP receptor differ from those of the human PP receptor. A precedent for pharmacological differences between species equivalents has been described for the serotonin (5-hydroxytryptamine, 5-HT) receptor family in which rat 5-HT$_{1B}$ and human 5-HT$_{1D}$ receptors are pharmacologically distinct despite their extensive sequence identity (28). Additional studies are required to determine the extent to which PP receptors exhibit species heterogeneity with respect to pharmacological specificity.

NPY receptors, namely Y1 and Y2, are negatively coupled to adenylate cyclase (19, 20). Accordingly, we tested the ability of human PP and human NPY to influence cAMP production in HEK 293 cells stably transfected with the human PP receptor. Neither peptide influenced basal cAMP levels, but both peptides dose-dependently inhibited forskolin-stimulated cAMP accumulation with a maximum inhibition of ≈50% observed (Table 2). In agreement with the binding data, human PP was a more potent inhibitor than human NPY. The estimated potency of each peptide to inhibit cAMP accumulation was greater than expected on the basis of their affinities for the human PP receptor, indicating that only fractional receptor occupation is required to elicit a maximum response. Thus, the human PP receptor is similar to both Y1 and Y2 receptors in that they are all negatively coupled to adenylate cyclase.

Sequence Comparison of NPY Receptor Family Members. Human and rat PP receptors are 80% and 74% identical at the nucleotide (coding region) and amino acid levels, respectively (Fig. 3). The transmembrane regions have the highest level of amino acid identity (84%), whereas the lowest levels are observed in the N-terminal (52%) and C-terminal (62%) regions.

Table 1. Peptide affinities for stably expressed PP receptors

Peptide	IC_{50}, nM	
	Human PP receptor	Rat PP receptor
Human PP	1.9 ± 0.4	0.3 ± 0.1
Bovine PP	2.3 ± 0.4	Not tested
Human [Pro^{34}]PYY	4.6 ± 1.4	7.4 ± 2.8
Rat PP	139.0 ± 39.0	0.4 ± 0.1
Human PYY	>1000	>1000
Human NPY	>1000	>1000

Shown are IC_{50} values for peptide inhibition of ^{125}I-hPP binding to human PP receptors and ^{125}I-rPP binding to rat PP receptors. Each value represents the mean ± SEM of between three and six separate experiments.

Table 2. Inhibition of forskolin-stimulated cAMP accumulation in HEK 293 cells expressing the human PP receptor

Condition	% cAMP accumulation
Forskolin (10 μM)	100
Forskolin + hPP (0.1 nM)	72 ± 3
Forskolin + hPP (10 nM)	50 ± 1
Forskolin + hNPY (10 nM)	80 ± 2
Forskolin + hNPY (1 μM)	47 ± 4

Inhibition of forskolin-stimulated cAMP accumulation was studied in cells stably transfected with human PP receptor cDNA. cAMP levels obtained in the presence of forskolin (10 μM) alone were arbitrarily set at 100%. The numbers (mean ± SEM, n = 3) represent the percentage of the forskolin response observed in the presence of the indicated concentration of peptide. Basal and forskolin-stimulated cAMP levels were 7 ± 1 fmol per 10^3 cells and 68 ± 20 fmol per 10^3 cells, respectively (n = 3). Neither peptide inhibited forskolin-stimulated cAMP accumulation in untransfected cells.

The level of sequence identity between human and rat PP receptors is substantially lower than the level observed between human and rat Y1 receptors (19, 29), which might reflect the fact that PP is less conserved between species than NPY (6).

The deduced amino acid sequence of the human PP receptor is 43% identical overall to the human Y1 receptor (29) (Fig. 3), with the greatest conservation occurring in the transmembrane domains (53%). The N-terminal region and second extracellular loop of the receptor exhibit the lowest levels of identity. The sequence of the human Y2 receptor, which was recently identified by expression cloning (20) and has been cloned in our laboratory, is 33% and 31% identical to human PP and human Y1 receptors, respectively, at the amino acid level (Fig. 3). The Y2 receptor sequence shown in Fig. 3 was derived in our laboratory and, with the exception of four amino acids, is identical to sequence published by Rose *et al.* (20), suggesting the possible existence of allelic polymorphism.

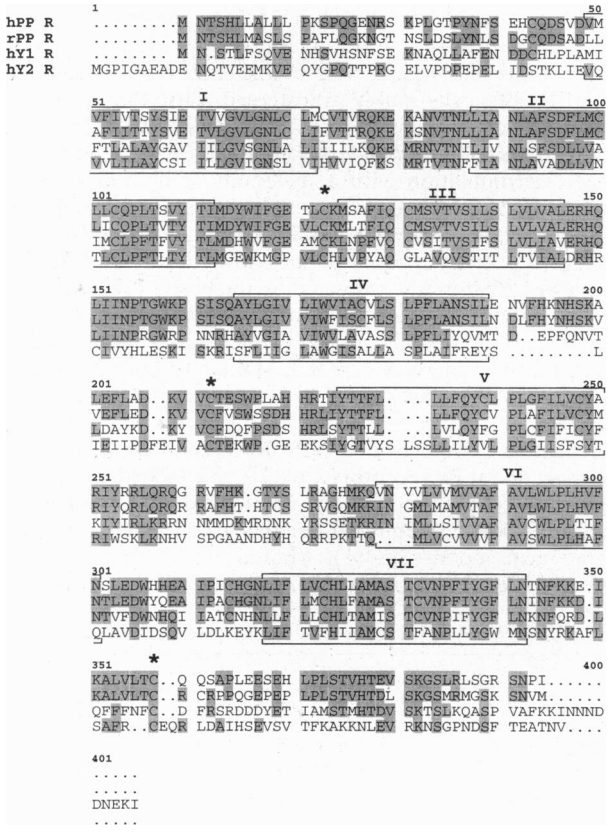

FIG. 3. Alignment of amino acid sequences of human PP receptor (first row), rat PP receptor (second row), human Y1 receptor (third row), and human Y2 receptor (fourth row) shown in one-letter code. Shaded areas designate amino acids that are identical in two or more sequences. The putative transmembrane domains are boxed, and the conserved cysteine residues are indicated by asterisks.

There are several potential N-glycosylation sites within both the human and rat PP receptors, of which three are located in the N-terminal region and one is located in the second extracellular loop. Cysteine residues located in the first and second extracellular loops, which have been implicated in intramolecular disulfide bond formation in other G-protein-coupled receptors (30), are conserved in both human and rat PP receptors. A third cysteine residue, which may be involved in anchoring the cytoplasmic tail of the receptor into the plasma membrane (31), is also conserved in both human and rat PP receptors.

It has been demonstrated that acidic residues in the extracellular loops of the human Y1 receptor are important for agonist binding, possibly through an electrostatic interaction with basic residues of NPY (32). Mutagenesis analysis has identified Asp^{104}, Asp^{194}, Asp^{200}, and Asp^{287} as essential for agonist binding to the Y1 receptor (32). Interestingly, all four acidic residues are conserved in the human PP receptor as Asp^{105}, Asp^{197}, Glu^{203}, and Asp^{289}. The same is true for the rat

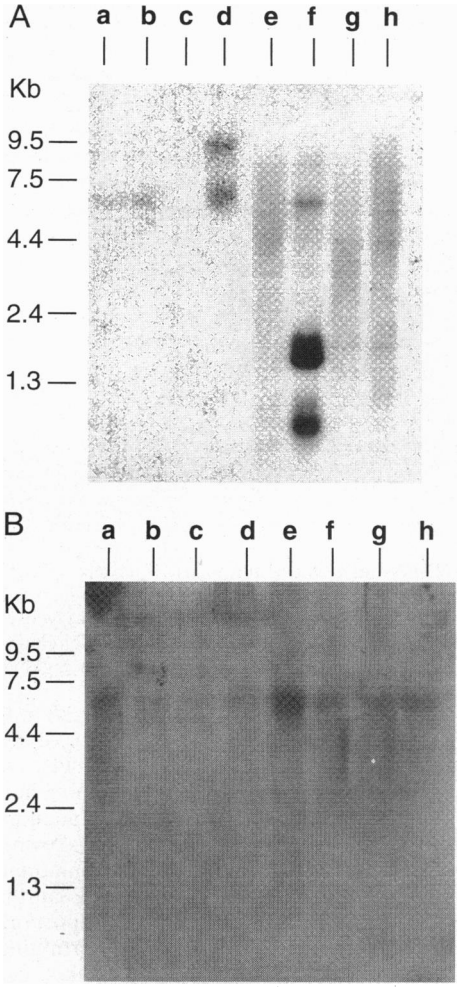

FIG. 4. Tissue distribution of human PP receptor mRNA by Northern blot analysis. Both human multiple tissue blot (*A*) and human brain blot (*B*) were hybridized with a full-length human PP receptor cDNA. The autoradiograms were exposed for 2 days (*A*) or 7 days (*B*) with two intensifying screens at −80°C. Lanes a–h in *A* represent heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, respectively. Lanes a–h in *B* represent amygdala, caudate nucleus, corpus callosum, hippocampus, hypothalamus, substantia nigra, subthalamic nucleus, and thalamus, respectively.

PP receptor, except that Asp^{200} is replaced by Val^{203}. Asp^{205} of the human Y1 receptor, an amino acid that, when mutated, leads to a 5-fold reduction in receptor affinity for NPY (32), is conserved only in the rat PP receptor.

Tissue Distribution of Human PP Receptor mRNA. High-stringency Northern blot analysis was used to determine the tissue distribution of human PP receptor mRNA (Fig. 4). The blots depicted in Fig. 4 were probed with the full-length coding region of human PP receptor cDNA. Identical results were obtained with cDNA probes specific for other regions of the receptor (data not shown). Three transcripts (1.2, 2.1, and 6.0 kb) were expressed in skeletal muscle, whereas the 6.0-kb transcript was detected in heart and lung. An additional 9.0-kb transcript was detected in lung. Only the 6.0-kb transcript was detected in human brain tissue, with the highest level of expression observed in the hypothalamus and lower levels of expression observed in the amygdala and thalamus. The presence of PP receptor mRNA in peripheral tissues and brain regions not generally considered to be targets for PP suggests that the peptide has a broader functional role than is currently recognized.

Concluding Remarks. The cloning and functional expression of cDNAs described in the present study conclusively demonstrates the existence of G-protein-coupled receptors in brain and peripheral tissues that selectively bind PP. This discovery represents an important step toward understanding the biological properties of PP and provides a valuable tool for the development of pharmacological agents that mimic or antagonize PP.

Note. During submission of this manuscript, the sequence of the human PP receptor was published by Bard *et al.* (33).

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