Neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) are structurally related peptides found in all higher vertebrates. NPY is expressed exclusively in neurons, whereas PYY and PP are produced primarily in gut endocrine cells. Several receptor subtypes have been identified pharmacologically, but only the NPY/PYY receptor of subtype Y1 has been cloned. This is a heptahelix receptor that couples to G proteins. We utilized Y1 sequence information from several species to clone a novel human receptor with 43% amino acid sequence identity to human Y1 and 53% identity in the transmembrane regions. The novel receptor displays a pharmacological profile that distinguishes it from all previously described NPY family receptors. It binds PP with an affinity (Kᵢ) of 13.8 nM, PYY with 1.44 nM, and NPY with 9.9 nM. Because these data identify the receptor as primarily a PP receptor, we have named it PP1. In stably transfected Chinese hamster ovary cells the PP1 receptor inhibits forskolin-stimulated cAMP synthesis. Northern hybridization detected mRNA in colon, small intestine, pancreas, and prostate. As all three peptides are present in the gut through either endocrine release or innervation, all three peptides may be physiological ligands to the novel NPY family receptor PP1.

Pancreatic polypeptide (PP) forms a family of 36-amino acid peptides together with neuropeptide Y (NPY) and peptide YY (PYY) (Larhammar et al., 1993). PP was the first of these to be discovered (Kimmel et al., 1968), but in evolutionary terms it seems to be the most recent member and probably arose by duplication of the PYY gene in early tetrapods (Larhammar et al., 1993). PP is exclusively localized to subsets of endocrine cells in the pancreas and inhibits pancreatic secretion, gall bladder contraction, and gut motility (see Hazelwood (1993) for review). PYY is expressed in PP cells and in somatostatin cells (Böttcher et al., 1993; Upchurch et al., 1994) as well as in endocrine cells of the large intestine (El-Salhy et al., 1983; Lundberg et al., 1982), has similar actions to PP, and, in addition, redistributes blood flow in gut vessels (see Laburthe (1990) for review). Both peptides are released into the circulation in response to a meal (see Hazelwood (1993)). In contrast, NPY is present in the central nervous system but is involved in gastrointestinal function through potent induction of feeding in the hypothalamus.

The NPY family peptides exert their actions via heptahelix (seven-transmembrane region) receptors coupled to G-proteins. Several receptor subtypes have been identified by their ability to bind NPY, PYY, PP, and derivatives of these peptides (Gehlert, 1994). Both NPY and PYY bind to the Y1 and Y2 receptors, while the Y3 receptor binds only NPY. The hypothalamic feeding receptor seems to be distinct from all of these (see Gehlert (1994)). An additional receptor has been described that displays a preference for PYY over NPY and is found in the rat small intestine (Laburthe et al., 1986) and in dog adipocytes (Castan et al., 1992), where it mediates reduction of lipolysis. PP does not bind to any of these subtypes but seems to have a unique receptor in dog intestinal mucosa (Gilbert et al., 1988; Gilbert et al., 1986), rat phaeochromocytoma PC12 cells (Schwartz et al., 1987), and rat adrenal cortex and medulla (Whitcomb et al., 1992) as well as in rat vas deferens (Jørgensen et al., 1990) and rat brain area postrema (Whitcomb et al., 1990). Finally, there is a PP-fold-recognizing receptor located in the distal colon in rabbit (Ballantyne et al., 1993) that binds all three peptides. While the discovery of selective peptide agonists has allowed a preliminary receptor classification, the lack of specific receptor antagonists has made functional studies difficult. For instance, it is unclear which receptor mediates the feeding induction reported for human PP in rats (Clark et al., 1984) and dogs (Inui et al., 1991).

To date only the Y1 receptor has been cloned. Cloning of additional receptor subtypes would be helpful to determine their preferences for the three endogenous peptides and to distinguish their physiological roles. The object of the present investigation was to isolate DNA clones encoding additional members of the NPY receptor subfamily. For this purpose we designed degenerate PCR primers based upon the Y1 receptor sequences from human (Herzog et al., 1992; Larhammar et al., 1992), rat (Eva et al., 1990), mouse (Eva et al., 1992), and Xenopus laevis (Blomqvist et al., 1995). This approach allowed the cloning of a novel receptor that has a higher degree of amino acid sequence identity to the Y1 receptor than to other heptahelix receptors. We also describe functional expression of this receptor to identify it as a PP-prefering receptor, hence named PP1.
EXPERIMENTAL PROCEDURES

Cloning of a Human Pancreatic Polypeptide Receptor

Cloning into Expression Vector—As no suitable restriction sites were available flanking the receptor gene for cloning into the expression vector, two oligonucleotides were used as PCR primers to generate a fragment containing the entire coding region. The 5’ primer contained a HindIII cloning site and the sequence CCG GTA AGC TTC CCG CGT CAT CCC TCA AGT GTA TC, and the 3’ primer had an EcoRI cloning site and the sequence GTT CAT CCC TCA AGT GTA TC. The PCR was run with Vent DNA polymerase (Biolabs) and the done Hubert as a template under the following conditions: 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C for 25 cycles. An aliquot of the PCR reaction was separated on an agarose gel and displayed the expected product of 1.25 kb. The remainder of the reaction was phenol-extracted and cut with HindIII-EcoRI, and the fragment was purified on an agarose gel and ligated into the expression vector pT98 (J. Hansen et al., 1990) to give the done Hubert-pTEJ. This clone was completely sequenced to ascertain identity to the genomic done.

Transient Transfection Protocol—COS1 African green monkey kidney cells (COS-1) were seeded at a density of 1×10⁵ cells/150-mm dish and incubated for 48 h at 37°C. Each dish was transfected with 600 μl of Lipofectace (Life Technologies, Inc.) containing 25 μg of Hubert-pTEJ according to kit protocol. Cells plus DNA/Lipofectace mixture were incubated for 6 h. Cells were harvested in PBS 48 h after transfection and pelleted by centrifugation.

Binding Assays—The homologous binding studies were conducted as described previously (Gehlert et al., 1992). Cell pellets were resuspended in a lysis medium (25 mM HEPES containing 2.5 mM CaCl₂, 1 mM MgCl₂, and 2 g/liter Bactaid). Saturation experiments were performed in a final volume of 200 μl containing various concentrations of 125I-pYY (SA 2200 Ci/mmol, DuPont NEN) and 5-10 μg of protein for 2 h at room temperature. Nonspecific binding was defined as the amount of radioactivity remaining bound to the cell homogenates after incubation in the presence of 1 μM unlabeled human PP (hPP). In competition studies, various concentrations of the peptides (hPP, hPYY, hNPY, porcine [Leu²-Pro³]-hPYY, porcine NPY2-36, and porcine NPY13-36) (Peninsula, Belmont, CA, or Bachem, King of Prussia, PA) were included in the incubation mixture along with [125I]-hPYY. Incubations were terminated by rapid filtration through GF/C filters (Wallac, Uithoorn, MD), which had been preoaked in 0.3% polyethyleneimine (Sigma), using a TOMTEC (Orange, CT) cell harvester. The filters were washed with 5 ml of 50 mM Tris (pH 7.4) at 4°C and rapidly dried at 60°C. The dried filters were treated with MetaLex A (Wallac) and melt-on scintillator sheets, and the radioactivity retained on the filters was counted using the Wallac 1202 Beta Plate counter. The results were analyzed using the Prism software (Graphpad, San Diego, CA) or the Cheng-Prushoff equation.

Protein concentrations were measured using Coomassie Protein Assay Reagent (Pierce) with bovine serum albumin for standards.

cAMP Assay—A cell line with stable P1 expression was obtained by transfection of Chinese hamster ovary cells with Hubert-pTEJ. The cAMP was assayed in whole cells treated for 20 min at 37°C with 100 μM insulin and 100 μM t-butylxanthines. Cells were incubated with 15 μM forskolin and various concentrations of hPP, hPYY, and hNPY at 15 min at 37°C. Reactions were terminated by the addition of EDTA to 0.4 μM and heating in a boiling water bath for 4 min. Sample buffer containing cAMP was removed and lyophilized. cAMP was quantitated using radioimmunossay (Amersham Corp.). Protein content of each well was measured using the Coomassie Protein Assay Reagent (Pierce) with bovine serum albumin as the standard.

RESULTS

Isolation of a Y1-related Rat PCR Product—To generate primers for PCR, we analyzed the sequences for the Y1 receptor from several species. Several degenerate primers were designed and used for PCR on rat genomic DNA. Two of the primers corresponding to TM2 and TM7 generated a product of the expected size. The fragment was cloned; one clone was sequenced and found to have higher sequence identity to the Y1 receptor than to all other receptor sequences.

Isolation of a Full-length Human Homologue—A human genomic library was screened under conditions of high stringency with the rat PCR product as a probe. Many clones hybridized, and six of the most strongly hybridizing ones were analyzed further. Five nonidentical clones contained the same hybridizing fragments in a Southern blot, while the sixth clone had a hybridization pattern indicating that it was truncated...
near the hybridizing segment but contained the same gene as the other five. Fragments of appropriate sizes were subcloned, and a restriction map was constructed. The subclone Hubert of 1.45 kb was found by sequencing to contain the entire coding region of a receptor with high identity to the rat PCR product. This clone encodes a heptahelix (7-TM) receptor of 375 amino acids (Fig. 1). It has greater amino acid sequence identity to the Y1 receptors (Fig. 2) than to any other receptor with 53% identity in the transmembrane regions and 43% overall identity. The closest non-Y1 receptor is the dog gastrin receptor (Kopin et al., 1992) with an overall identity of about 30% (Fig. 2).

The novel receptor gene lacks the intron immediately after TM5 that is present in the Y1 receptor genes in all four species characterized to date. The nucleotide sequence identity to the human Y1 sequence is 58%.

The receptor protein deduced from the nucleotide sequence displays many of the characteristic features of heptahelix receptors (Figs. 1 and 2). The amino terminus has three potential glycosylation sites, and a fourth potential site is present in each of the three extracellular loops, presumably for N-linked glycosylation. Diamonds show four extracellular cysteines and one intracellular cysteine.

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The sequence similarity to Y1 is most prominent in the transmembrane regions, but the loops also show blocks of resemblance. The sequenced portion of the gene extends 180 base pairs beyond the termination codon, but no polyadenylation signal was found in agreement with the large size of the mRNA (see below).

**Southern Hybridization**—A single band corresponding to the isolated receptor gene was observed at high stringency (not shown), suggesting that the human genome contains a single PP1-receptor gene.

**Northern Hybridization**—The expression of receptor mRNA in various human organs and brain regions was investigated by Northern hybridization. Among the organs (Fig. 3), colon, small intestine, pancreas, and prostate showed a band in the range 6–7 kb. All of the other peripheral organs gave no signal. In the nervous system, faint signals were observed in cerebellum, medulla, and spinal cord after long exposure (not shown).

**Binding Properties of the Novel Human Receptor**—The coding portion of the clone Hubert was cloned into the expression vector pTEJ-8 and transfected into COS1 cells. Membranes prepared from these cells exhibited concentration-dependent binding of $^{125}$I-pPYY (Fig. 4). This radioligand identified a single class of high-affinity binding sites with an affinity constant ($K_d$) of $148 \pm 62\, \mu M$ ($n = 3$, S.E.) for $^{125}$I-pPYY and $B_{max}$ of $258 \pm 46\, \mu M$ protein. Nontransfected COS1 cells exhibited no specific binding of $^{125}$I-pPYY (data not shown). Competition experiments were performed using PYY, PP, and various peptide analogues (Fig. 5). Both hPP and hPYY were potent inhibitors of $^{125}$I-pPYY binding with inhibition constants ($K_i$) of $13.8 \pm 0.4\, \mu M$ ($n = 4$, S.E.) and $1.44 \pm 0.2\, \mu M$ ($n = 4$, S.E.), respectively. NPY was less potent, with a $K_i$ of 9.88
The difference for PYY between $K_d$ (148 pM) and $K_i$ (1.44 nM) is probably because porcine PYY was used for the former and human PYY for the latter, and the two species differ at two amino acid positions (Larhammar et al., 1993). The Y1-selective analog h[Leu$^{31}$-Pro$^{34}$]NPY was slightly less potent than NPY, with a $K_i$ of 212 ± 2.0 nM ($n = 4, ±S.E.$) and substantially less potent than PYY and PP. Also porcine NPY2–36 was slightly less potent than intact NPY with a $K_i$ of 42.2 ± 1.6 nM ($n = 4, ±S.E.$). The Y2-selective fragment, porcine NPY13–36, had very low potency, with a $K_i$ of 139 ± 4 nM ($n = 4, ±S.E.$). Several unrelated peptides were also tested and did not significantly affect binding at 1 μM concentrations.

**DISCUSSION**

Binding studies to different tissue preparations and cell lines have demonstrated the existence of several distinct receptor subtypes that bind NPY family peptides and peptide analogues. The molecular and physiological characterization of these receptors requires access to molecular clones that can be used for functional expression in cell lines and design of specific DNA and RNA probes. So far only the Y1 receptor has been cloned. We have used molecular biology approaches to find clones for additional receptor subtypes related to Y1 and describe here one such clone that displays greater homology to the Y1 receptor than to any other G-protein-coupled receptor. Because the novel receptor preferentially binds PP among the NPY family peptides, we call the receptor PP1.

The human PP1 receptor consists of 375 amino acids with 53% identity to the human Y1 receptor in the TM regions. This degree of identity is similar to that between different subtypes of tachykinin or somatostatin receptors in the TM regions. The overall identity to hY1 is 43%. The PP1 receptor shares several features with Y1 such as three amino-terminal glycosylation sites and four extracellular cysteines. Intracellular loops 1 and 2 have multiple identical positions; loop 1 has seven out of ten identities, and in loop 2 the first nine amino acids are identical. This motif, ERHQL1INP, is also conserved among all four known Y1 sequences (Blomqvist et al., 1995). It will be interesting to see whether other NPY family receptor subtypes have...
the same motif. The sequence similarity in the intracellular loops is consistent with the finding that the PP1’s messenger response, namely inhibition of forskolin-stimulated cAMP synthesis (Fig. 6), is similar to that of Y1.

A recent study of the human Y1 receptor by site-directed mutagenesis suggested four acidic residues as points of interaction with basic side chains in NPY, namely Asp-104, Asp-194, Asp-200, and Asp-289 (Walker et al., 1994). We have previously shown that three of these positions are negatively charged also in the Xenopus laevis Y1 receptor (Blomqvist et al., 1995), whereas the position corresponding to Asp-194 is a glycine. The PP1 receptor presented here has negatively charged side chains in all four corresponding positions, namely Asp-105, Asp-197, Glu-203, and Asp-289. The similar pattern in negatively charged residues might indicate that PP and PYY bind this receptor in a manner similar to NPY binding to Y1. However, because NPY binds less strongly to PP1 than to Y1, there clearly may be additional structural aspects that diminish NPY binding to the PP1 receptor.

While many heptahelix receptor genes lack introns in their coding regions, the Y1 gene was found to have a small intron immediately after the segment encoding TM5 (Eva et al., 1992; Herzog et al., 1992; Larhammar et al., 1992). The human PP1 receptor gene described here lacks this intron as does the rat genomic fragment generated with PCR that was used to isolate the human PP1 gene. Evolutionary studies may show whether the intron was present in the ancestral NPY family receptor gene. Southern hybridizations to human genomic DNA at high stringency suggest a single PP1 receptor gene.

The functional expression binding studies of the PP1 receptor revealed a high affinity for hPP with a $K_i$ of only 13.8 nM. The PP1 receptor also exhibits high affinity for hPYY (1.44 nM) and hNPY (9.9 nM). No previous reports in the literature have shown that three of these positions are negatively charged also in the Xenopus laevis Y1 receptor (Blomqvist et al., 1995), whereas the position corresponding to Asp-194 is a glycine. The PP1 receptor presented here has negatively charged side chains in all four corresponding positions, namely Asp-105, Asp-197, Glu-203, and Asp-289. The similar pattern in negatively charged residues might indicate that PP and PYY bind this receptor in a manner similar to NPY binding to Y1. However, because NPY binds less strongly to PP1 than to Y1, there clearly may be additional structural aspects that diminish NPY binding to the PP1 receptor.

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