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The Peptide YY-Preferring Receptor Mediating Inhibition of Small Intestinal Secretion Is a Peripheral Y_2 Receptor: Pharmacological Evidence and Molecular Cloning

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ABSTRACT

A peptide YY (PYY)-preferring receptor [PYY > neuropeptide Y (NPY)] was previously characterized in rat small intestinal crypt cells, where it mediates inhibition of fluid secretion. Here, we investigated the possible status of this receptor as a peripheral Y_2 receptor in rats. Typical Y_2 agonists ($PYY_{3–36}$, $NPY_{3–36}$, $NPY_{13–36}$, C2-NPY) and very short PYY analogs (*N*-α-Ac-$PYY_{22–36}$ and *N*-α-Ac-$PYY_{25–36}$) acting at the intestinal PYY receptor were tested for their ability to inhibit the binding of ^{125}I-PYY to membranes of rat intestinal crypt cells and of CHO cells stably transfected with the rat hippocampal Y_2 receptor cDNA. Similar PYY preference was observed and all analogs exhibited comparable high affinity in both binding assays. The same held true for the specific Y_2 antagonist BIIE0246 with a K_i value of 6.5 and 9.0 nM, respectively. BIIE0246 completely abolished the inhibition of cAMP production by PYY in crypt cells and transfected CHO cells. Moreover, the antagonist 1) considerably reversed the PYY-induced reduction of short-circuit current in rat jejunum mucosa in Ussing chamber and 2) completely abolished the antisecretory action of PYY on vasoactive intestinal peptide (VIP)-induced fluid secretion in rat jejunum in vivo. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) experiments showed that Y_2 receptor transcripts were present in intestinal crypt cells (3×10^2 molecules/100 ng RNA_T) with no expression in villus cells, in complete agreement with the exclusive binding of PYY in crypt cells. Finally, a full-length Y_2 receptor was cloned by RT-PCR from rat intestinal crypt cells and also from human small intestine. We conclude that the so-called PYY-preferring receptor mediating inhibition of intestinal secretion is a peripheral Y_2 receptor.

Peptide YY, neuropeptide Y, and pancreatic polypeptide are members of the PP-fold family of peptides (Michel et al., 1998; Gehlert, 1998). They share important structural and biological similarities but originate from different sources (Gehlert, 1998; Cerda-Reverter and Larhammar, 2000). PYY is an intestinal hormone released by distal small intestine and colon, whereas NPY is a neurotransmitter in the brain and at the periphery (Gehlert, 1998). PP is a circulating peptide produced by PP-cells in the endocrine pancreas (Gehlert, 1998).

It has long been known that a PYY receptor is present in the rat small intestinal epithelium (Laburthe et al., 1986). It was defined as PYY-preferring because it exhibits a ~10-fold higher affinity for PYY than for NPY (Laburthe et al., 1986). This receptor was shown to be negatively coupled to cAMP production (Servin et al., 1989) and to be located in crypt cells (Voisin et al., 1990). Thereafter, numerous reports showed a potent inhibition of intestinal electrolyte secretion by PYY (Cox et al., 1988; Voisin et al., 1990; Cox, 1998; Fu-Cheng et al., 1999), suggesting that agonists acting at this receptor may have potential antidiarrheal value (Laburthe, 1991; Playford and Cox, 1996). The inhibitory effects on cAMP production (Servin et al., 1989) and electrolyte secretion (Laburthe et al., 1982; Voisin et al., 1990; Eto et al., 1997) in intestine were also shown to be PYY-preferring with PYY being more potent than NPY. The PYY preference has recently been claimed as being a common characteristics of receptors in gastrointestinal epithelia (Holliday et al., 2000). Although characterized at the biochemical level as a 44-kDa glycoprotein (Voisin et al., 1991), the intestinal PYY receptor

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ABBREVIATIONS: PYY, peptide YY; NPY, neuropeptide Y; PP, pancreatic polypeptide; CHO, Chinese hamster ovary; p, porcine; r, rat; VIP, vasoactive intestinal peptide; KRB, Krebs-Ringer buffer; RT-PCR, reverse transcription-polymerase chain reaction; TBE, Tris-borate EDTA; bp, base pair(s); TTX, tetrodotoxin; BIIE0246, (*S*)-*N*2-[[1-[2-[4-[(*R*,*S*)-5,11-dihydro-6(6h)-oxodibenz[*b*,*e*]azepin-11-yl]-1-piperazinyl]-2-oxoethyl] cyclopentyl] acetyl]-*N*-[2-[1,2-di-hydro-3,5-(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamid.

has not been yet cloned. Convincing evidence for the existence of such a receptor is still lacking thereby.

Several receptor subtypes that bind PYY, NPY and/or PP have been cloned (Michel et al., 1998; Ingenhoven and Beck-Sickinger, 1999; Laburthe et al., 1999). They are heptahelical G protein-coupled receptors and are referred to as Y receptors (Michel et al., 1998). Y_1, Y_2, and Y_5 receptors have similar affinity to NPY and PYY, whereas Y_4 receptor seems to be specific for PP (Voisin et al., 2000) although there was initial confusion regarding its pharmacological profile (Bard et al., 1995; Lundell et al., 1995, 1996; Yan et al., 1996). A Y_6 receptor has been cloned in mice (Weinberg et al., 1996) but seems to be absent in rats (Burkhoff et al., 1998). Finally, a putative Y_3 receptor claimed to be specific for NPY has been proposed in rat cardiac ventricular membranes, with NPY_{18-36} being a competitive antagonist (Balasubramaniam and Sheriff, 1990). However, this putative subtype remains to be cloned (Michel et al., 1998). Therefore the pharmacology of cloned Y receptors is not fully characterized, whereas potential additional Y receptors, not yet cloned, have been suggested (Blomqvist and Herzog, 1997).

The pharmacology of the intestinal PYY receptor is clearly different from that of the Y_4 receptor specific for PP or the putative Y_3 receptor specific for NPY (Laburthe et al., 1999). We demonstrated previously that it is not a Y_5 receptor (Goumain et al., 1998a). Because it binds with high affinity C-terminal PYY fragments (Laburthe et al., 1986), including PYY_{22-36}, PYY_{25-36}, and analogs (Balasubramaniam et al., 2000), the intestinal PYY receptor is not a Y_1 receptor, which requires the presence of the N terminus of PYY or NPY for high-affinity binding (Ingenhoven et al., 1999). The ability of the intestinal PYY receptor to accept deletion of the N-terminal domain of PYY makes it Y_2-like because Y_2 receptors can bind NPY_{3-36} or NPY_{13-36} with high affinity (Ingenhoven et al., 1999). However, there are arguments disproving this view: 1) after initial cloning of Y_2 receptor in the central nervous system, Y_2 receptor mRNAs were found in several areas of the brain, but no transcripts could be detected at the periphery (Gerald et al., 1995; Rose et al., 1995; Gehlert et al., 1996); 2) the PYY preference displayed by the intestinal receptor (Laburthe et al., 1986) was not consistently reported for the cloned human Y_2 receptors (Gehlert et al., 1996) or the native Y_2 receptors (Gehlert, 1998; Ingenhoven and Beck-Sickinger, 1999); 3) recent RT-PCR experiments identified Y_2 receptor mRNA in both intestinal epithelial crypt cells and villus cells (Goumain et al., 1998b), whereas PYY binding occurs exclusively in crypt cells (Voisin et al., 1990).

In this context, the present study has been designed to determine whether or not the intestinal PYY receptor is a peripheral Y_2 receptor. We took advantage of two recent advances in this area: 1) the cloning of a rat Y_2 receptor from hippocampus (St Pierre et al., 1998) and 2) the development of a specific Y_2 antagonist (Doods et al., 1999). We show here that the pharmacological profiles of PYY-preferring receptor from rat crypt cells and rat hippocampal Y_2 receptor stably transfected in CHO cells are identical. We further demonstrated that the Y_2 receptor antagonist BIIE0246 blocks PYY-induced inhibition of cAMP production in isolated crypt cells, PYY-induced decrease of short-circuit current in rat jejunum mucosa, and PYY-induced inhibition of fluid secretion in rat jejunum in vivo. Finally, we showed by quantitative RT-PCR that Y_2 transcripts are exclusively expressed in crypt cells of the jejunum and we cloned a full-length Y_2 receptor cDNA from rat crypt cells that exhibits 100% identity with the sequence of the rat hippocampal Y_2 receptor cDNA.

Experimental Procedures

Materials. Male Wistar rats weighing 250 to 280 g were obtained from Iffa-Credo (Iffa-Credo, Les Oncins, L'Arbresle). They were fed with standard laboratory chow and had free access to tap water. Animal care was according to the Recommendations of the local committee and the National Institutes of Health *Guidelines for the Care and the Use of Laboratory Animals, 1985.* The Chinese hamster ovary (CHO) cells were purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and cultured in Ham's F12 nutrient mixture (Invitrogen, Leek, The Netherlands) supplemented with 10% fetal calf serum (v/v) (Invitrogen), 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Cells were passaged every 3 days in 25-cm^2 plastic culture flasks and used between the 4th and 25th passages. Analogs and fragments of rat (r) NPY_{1-36}, porcine (p) NPY_{3-36}, $rNPY_{13-36}$, $r[Leu^{31},Pro^{34}]NPY_{1-36}$, $r[D\text{-}Trp^{32}]NPY_{1-36}$, $pPYY_{1-36}$, $pPYY_{3-36}$, rPP_{1-36}, hPP_{1-36}, and the Y_1 antagonist/Y_4 agonist 1229U91 (also known as homodimeric Ile-Glu-Pro-Dpr-Tyr-Arg-Leu-Arg-Tyr-$CONH_2$ or GW1229) were purchased from Neosystem (Strasbourg, France) or Peninsula Laboratories (Belmont, CA). Rat *N*-α-acetyl-PYY_{22-36} and *N*-α-acetyl-PYY_{25-36} were synthesized as described previously (Balasubramaniam et al., 2000). The Y_2-specific antagonist BIIE0246 was provided by Boehringer Ingelheim Pharma (Biberach, Germany) (Doods et al., 1999). Iodine-125 was incorporated into the tyrosine 36 residue of pPYY using the chloramine T method and purified as described previously (Voisin et al., 1991). The specific activity was assumed to be of the theoretical value (2200 Ci/mmol). The eucaryotic vector pcDNA3 was purchased from Invitrogen (Cergy Pontoise, France).

Stable Transfection of CHO Cells and Related Protocols. Full-length rat Y_2 receptor cDNA cloned in hippocampus (St. Pierre et al., 1998) was provided by author H. H. The cDNA was subcloned into the pcDNA3 expression vector, which contains the selectable geneticin gene. The recombinant plasmid was transfected into the CHO cell line by electroporation. Briefly, CHO cells were rinsed in phosphate-buffered saline, removed from the culture dish using 0.5-ml versene/trypsin (5 min at 37°C) and pelleted by centrifugation. CHO cells were resuspended in phosphate-buffered saline at a concentration of 10^6 cells/ml and then 500 μl of cell suspension (0.5×10^6 cells) were transferred in an electroporation cuvette together with 15 μg of rat Y_2 cDNA/pcDNA3 and 15 μg of salmon sperm DNA as carrier. After gentle shaking for 5 min at 4°C, electroporation was performed (330 V, 500 μF) using a Electroporator II (Invitrogen). Transfected cells were then resuspended in culture medium, transferred into 150-mm Petri dishes, and incubated for 48 h at 37°C. Two days after transfection, cells were selected through the addition of G418 Geneticin, at a final concentration of 1 mg/ml for 3 weeks. Resistant transfected cells were cloned by limiting dilution. Briefly, transfected CHO cells suspension was distributed to a microtest 96-well plate, at a mean ratio of 0.25 cell/well. Cells grown in wells observed to initially contain one cell were subsequently transferred to larger culture vessels. Among the 13 clones obtained, clone 11 was selected on the basis of its binding capacity for PYY, which was similar to that found in rat intestinal crypt cells (see *Results*). Clone 11 cells were then cultured in standard culture medium (see above) supplemented with G418 Geneticin (400 μg/ml) and were routinely passaged every 3 days. Stably transfected cells (clone 11) were grown in 75-cm^2 plastic culture flasks for 4 to 5 days. Cell membranes were prepared for binding experiments as follows: cells were washed three times with 0.13 M phosphate-buffered saline, pH 7.4, harvested using a rubber policeman, and centrifuged at 2000*g* for 5 min at 4°C.

The cell pellet was then exposed for 30 min to hypoosmotic 5 mM HEPES buffer, pH 7.4, as described previously (Voisin et al., 1990). The resulting broken cell suspension was centrifuged at 20,000 g for 15 min, washed with 20 mM HEPES buffer, pH 7.4, pelleted and stored at −80°C until use. This particulate fraction will be referred to as membrane preparation.

Transient Transfection of CHO Cells with the Human Y_2 Receptor cDNA. CHO cells were transiently transfected with a full-length human Y_2 receptor cDNA inserted in pcDNA3 expression vector, by using an electroporation method as described above (see *Stable Transfection of CHO Cells*). Transfected cells were then resuspended in culture medium, transferred into 150-mm Petri dishes, and cultured for 48 h at 37°C. Cell membranes were then prepared for binding experiments as described above.

Preparation of Intestinal Epithelial Cells and Related Protocols. Jejunum was removed after decapitation of rats and crypt cells were separated from villus cells by shaking the everted intestine for successive periods in a dispersing solution containing EDTA as described (Voisin et al., 1990). Cell fractions were first characterized by optical microscopy. Villus sheets were morphologically different from crypts, which had the shape of sticks of cells. Villus cells were then characterized by their brush border-associated alkaline phosphatase activity (Voisin et al., 1990). Enzyme activity was six times higher in villus cells than in crypt cells, as reported previously (Voisin et al., 1990) [i.e., 886 ± 77 versus 146 ± 11 mU/mg of protein (five experiments)]. Proliferative crypt cells were identified by their ability to incorporate [methyl-^{3}H]thymidine (50 μCi), which had been injected to rats 2 h before death (Voisin et al., 1990). Crypt cells clearly incorporated 30 times more radioactivity than villus cells [i.e., 6830 ± 420 versus 210 ± 73 dpm/0.1 mg of protein (five experiments). Rat colonic epithelial cells were isolated as described previously (Laburthe et al., 1986). Cells were used immediately for RNA extraction (see below). The crypt cell fraction was also used to prepare crude membranes as described previously (Voisin et al., 1990). Briefly, crypt cells were homogenized with the use of a Waring blender in 0.25 M sucrose buffered with 10 mM triethanolamine, pH 7.5, containing 5 mM EDTA. After centrifugation at 2,600 g for 10 min, the supernatant was centrifuged at 20,000 g for 15 min. The resulting pellet was washed in 20 mM HEPES buffer, pH 7.5, repelleted, and stored at −80°C until use (Voisin et al., 1990).

Binding Assay. Binding of ^{125}I-peptide YY to membranes prepared from crypt cells or transfected cells was carried out as described previously (Voisin et al., 1990). Briefly, membranes (200 μg of protein/ml) were incubated at 15°C for 120 min in 250 μl of incubation buffer [20 mM HEPES, pH 7.4, 2% (w/v) bovine serum albumin] containing 0.05 nM ^{125}I-PYY with or without unlabeled PYY or other competitors. At the end of incubation, aliquots (150 μl) of membranes were mixed with 150 μl of ice-cold incubation buffer. Bound and free peptides were separated by centrifugation at 20,000 g for 10 min, and membrane pellets were washed twice with 10% (w/v) sucrose in 20 mM HEPES. All binding data were analyzed using PRISM 3.0 software (GraphPad Software, San Diego, CA). The constant K_i for the inhibition of ^{125}I-peptide YY binding by unlabeled competitors was calculated from the concentration of unlabeled competitor that produces 50% inhibition (IC_{50}) of the specific ^{125}I-peptide YY binding using the following relation: $K_i = IC_{50} \times [K_d / (K_d + L)]$ where K_d is the dissociation constant and L the concentration of ^{125}I-peptide YY. K_i values are given as geometric means with 95% confidence limits in parentheses from 3 to 15 experiments.

cAMP Assay. Isolated intestinal crypt cells at 200 μg protein/ml were incubated under continuous agitation for 45 min at 15°C in 0.5 ml of phosphate-buffered saline, pH 7.0, containing 1.4% (w/v) bovine serum albumin, 0.1% bacitracin, and 0.2 mM 3-isobutyl-1-methylxanthine (IBMX) as described previously (Servin et al., 1989). PYY or NPY were added together with the most potent physiological stimulant of cAMP production in enterocytes (e.g., VIP). The reaction was initiated by adding cells and was stopped after 45 min by adding 50 μl of 11 M perchloric acid. After centrifugation for 10 min at 4,000*g*, the cAMP present in the supernatant was succinylated, and its concentration was measured by radioimmunoassay as described previously (Laburthe et al., 1982). The same method was used for cAMP assay on CHO clone 11 cells, but cultured cells were incubated at a final concentration of 1.5×10^6 cells/ml, and forskolin was used to stimulate cAMP production.

Ussing Chamber. The jejunum (5 cm distal to the ligament of Treitz) from 24 h-fasted rats was dissected out and rinsed in cold KRB solution (see below) to remove luminal contents. The mesenteric border was carefully stripped off and the serosa and longitudinal muscle/myenteric plexus were stripped away using forceps. Intestine was then opened along the mesenteric border and placed between the two halves of a Ussing chamber (exposed area: 0.63 cm^2). The tissues were bathed with 6 ml of KRB solution on each side and maintained at 37°C. The KRB bicarbonate solution (115.4 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.6 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $CaCl_2$, and 10 mM glucose) was gassed with 95% O_2/5% CO_2 and kept at constant temperature of 37°C, pH 7.4. Electrogenic ion transport was monitored continuously as the short-circuit current (Isc) by using an automated voltage-clamp apparatus (DVC 1000; WPI, Aston, England) linked through MacLab 8 to a Macintosh computer. Every 50 s, the tissue was automatically clamped at ± 1 mV for 5 s to calculate the electrical conductance according to Ohm's law. Results are expressed as the intensity of the Isc (microAmperes/square centimeter) or as the difference (ΔIsc) between the Isc measured within 10 min after addition of the compounds and the basal Isc (measured just before addition of the compound). Stock solution of PYY, VIP, and BIIE0246 were prepared in 0.9% NaCl containing 0.3% bovine serum albumin to limit peptide adherence to glassware, stored frozen at −20°C and thawed immediately before use. Working solutions were prepared by serial dilution of the stock solution in KRB to achieve final concentration in the range 10^{-6} to 10^{-9} M in the Ussing chamber compartments. All solutions were added as 100-μl samples to the serosal bath.

In Vivo Studies. Jejunal ligated loops were prepared in 24 h-fasted rats as described in detail by Chariot et al. (2000). Briefly, in pentobarbital-anesthetized rats (50 mg/kg, intraperitoneal), closed loops of proximal jejunum (10 cm long) were tied off and filled at time 0 with 1 ml of 0.9% saline. Care was taken during the loop preparation to preserve the marginal artery of the bowel from damage. The loops were then returned to the abdomen. Their normal anatomic placement was maintained as far as possible, and the abdominal wall was closed. After 30 min, the rats were killed, the loops were cut off, and the amount of fluid remaining in the lumen was measured, which allowed us to calculate the net water flux. Under these conditions, jejunal net water fluxes were negative, indicating a net absorption, which will be designated below as basal net water flux. To evidence more easily the antisecretory effect of PYY, the basal absorption was counteracted with VIP (100 μg/kg/h) infused intravenously (saphenous vein) at 2.5 ml/h for 30 min (from t = 0 to t = 30 min). This concentration was chosen from previous experiments as producing a net secretion in our experimental conditions. The net water flux measured under VIP infusion is designated below as "VIP-stimulated net water flux". PYY was administered as an intravenous infusion of 300 pmol/kg/h beginning 15 min before starting the VIP infusion (from t = −15 to t = 30 min). To analyze the specific mechanisms involved in the effect of PYY, the Y_2 or Y_1 receptor antagonists (30 nmol/kg/h) were injected with PYY. These concentrations of antagonist drugs were chosen based on the data described in this article. Appropriate control groups were set up to determine the effect of the antagonists on basal net water flux. The effect of the antagonist was determined by comparing the net water flux with and without antagonist after infusion of saline, and of VIP + PYY, by analysis of variance followed by a Dunnett's test. Differences with $P < 0.05$ were considered significant.

RNA Extraction and Quantitative RT-PCR of Y_2 Receptors. Total RNA was extracted from rat epithelial crypt or villus cells using RNAxel reagent (Goumain et al., 1998b). Total RNA was

quantified at 260/280 nm, and the integrity of the samples was checked by 1% agarose gel electrophoresis. Aliquots were stored in sterile microcentrifuge tubes at −80°C until use. Quantitative RT-PCR of Y_2 receptors was developed as described previously (Rouet-Benzineb et al., 1999). Design of sense and anti-sense primers for rat Y_2 receptor was performed using the previously published rat brain Y_2 receptor cDNA sequence (St. Pierre et al., 1998). The rat Y_2 receptor primers are 5′-AAA TGG GTC CAG TTT TGT GCC-3′ (sense) and 5′-TGC CTT CGC TGA TGG TAA TGG-3′ (anti-sense). A specific internal DNA standard was obtained by amplification of foreign DNA fragment issued from the ampicillin resistance gene in the pcDNA3 plasmid using composite primers rY_2-Amp (sense, 5′-AAA TGG GTC CAG TTT TGT GCC **TCC AGA TTT ATC AG**-3′; antisense, 5′-TGC CTT CGC TGA TGG TAA TGG **CAA GAG CAA CTC GGT C**-3′). DNase-treated RNA_T (2 μg) from each individual cell fractions (crypt, villus, and colon) was reverse-transcribed into cDNA for 1-h incubation period at 37°C in a reaction medium composed of 30 μl of (final concentration) 1.5 μM oligo(dT_{15}), 0.5 mM dNTP, 13.3 U/μl Moloney murine leukemia virus reverse transcriptase (Invitrogen) in 10 mM Tris-HCl, pH 8.3, 75 mM KCl, and 0.6 mM $MgCl_2$. For each PCR, 1 μl of the first-strand cDNA and 1 μl of internal DNA standard (103 molecules) were added to 48 μl of a mixture containing 20 pmol/μl of each Y_2 primer; 200 μM each dATP, dCTP, dTTP, and dGTP; 50 mM KCl; 10 mM Tris-HCl, pH 9.0; 1.5 mM $MgCl_2$, 0.3 μl of [α-^{32}P]dCTP (3 μCi/nmol), and 0.5 U of *Thermus aquaticus* thermostable DNA polymerase. PCR amplification was performed using thermocycler set for 25 cycles of 1-min denaturation at 94°C, 1-min annealing at optimal Tm (60°C), and 1-min extension at 72°C. The last amplification was followed by a final 10-min elongation step at 72°C. PCR fragments were analyzed by electrophoresis in 4% polyacrylamide gel with 1× TBE (100 mM Tris, 90 mM boric acid, and 1 mM EDTA) as running buffer. The quantities of amplified internal standard or amplified target RNA_T in each tube were compared by autoradiography and scanning of bands with a Hewlett Packard ScanJet 6100C Scanner densitometer. Each Y_2 amplicon was characterized by specific restriction enzyme digestion using *Bgl*II enzyme. The expected fragments were resolved by 4% PAGE with 1× TBE in the presence of molecular mass DNA markers. Bands were cut off the gel and the radioactivity of each band was counted by liquid scintillation spectrometry. Bands were then quantified by comparison with the amplified internal standard. Primers for rat β-actin, taken as a control for housekeeping protein, were used.

Rat Intestinal Y_2 Receptor Cloning. A total of 10 μg of rat crypt DNase-treated RNA was reverse-transcribed as described above, and 5 μl of the resulting cDNA mixture was submitted to PCR using specific primers. Five oligonucleotides, chosen from rat brain Y_2 cDNA cloned sequence (St. Pierre et al., 1998), were used for PCR (see below) on rat crypt cDNA using the following conditions: 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for 25 cycles using *Taq* polymerase (ATGC, Noisy-le-Grand, France). PCR products including the expected full-length rat crypt Y_2 cDNA (1147 bp) were cloned using the pGEMT-Easy kit (Promega, Charbonnieres, France) and sequenced by Genome Express (Grenoble, France). Oligonucleotides primers used for RT-PCR-based cloning strategy of the rat intestinal Y_2 receptor were the following: oligo 1 (sense, 1–27) 5′-ATG GGC CCA TTA GGT GCA GAG GCA GAT-3′; oligo 2 (sense, 51–71) 5′-AGT GGA ACT CTA TGG GTC GGG-3′; oligo 3, (sense, 350–371) 5′-AAA TGG GTC CAG TTT TGT GCC-3′; oligo 4 (antisense, 791–770) 5′-TGC CTT CGC TGA TGG TAA TGG-3′; oligo 5 (antisense, 1147–1127) 5′-CTT ACA CGT TGG TGG CCT CTG 3′

Human Intestinal Y_2 Receptor Sequencing. A total of 100 ng of QUICK-clone cDNA (CLONTECH Laboratories, Palo Alto, CA) from human small intestine were submitted to PCR using specific human primers. Four oligonucleotides, chosen from the human brain Y_2 receptor cDNA sequence (Rose et al., 1995), were used for PCR under the following conditions: 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C for 35 cycles using *Taq* polymerase (ATGC). Two PCR products of 791 bp and 797 bp, including a 442-bp overlapping sequence, were sequenced by Genome Express (Grenoble, France). Oligonucleotides primers used for PCR were the following: oligo A (sense, 1–23) 5′-ATG GGT CCA ATA GGT GCA GAG GC-3′; oligo B (sense, 350–371) 5′-AAA TGG GTC CTG TCC TGT GCC-3′; oligo C (antisense, 791–770) 5′-TGC CTT CGC TGA TGG TAG TGG-3′; oligo D (antisense, 1147–1127) 5′-CTT AGA CAT TGG TAG CCT CTG-3′.

Results

In a first set of experiments, we compared the pharmacological profiles of PYY receptor in membranes from rat intestinal crypt cells and CHO cells stably transfected with the rat brain Y_2 receptor cDNA. Scatchard analysis of the competitive inhibition of ^{125}I-PYY binding to membranes by unlabeled PYY indicated that binding parameters were similar in the two membrane preparations [e.g., B_{max} = 150 ± 33 versus 377 ± 80 fmol/mg protein and K_d = 0.14 ± 0.03 versus 0.33 ± 0.07 nM, respectively ($n \geq 15$)]. Competitive inhibitions of tracer binding with native PYY or NPY showed similar PYY preference in both systems (Table 1). Moreover, $PYY_{3–36}$, $NPY_{3–36}$, $NPY_{13–36}$, or C2-NPY, which are considered typical Y_2 agonists (Gehlert, 1998), clearly exhibited similar high affinity for the rat brain recombinant Y_2 receptor and the rat crypt cell PYY receptor (Table 1). Conversely, *N*-α-Ac-$PYY_{22–36}$ and *N*-α-Ac-$PYY_{25–36}$, which are known to have high affinity to the rat cryptic PYY receptor (Balasubramaniam et al., 1993, 2000; Goumain et al., 1998a) and to behave as potent antisecretory agents in rat intestine (Balasubramaniam et al., 1993), also have high affinity for the rat brain Y_2 receptor (Table 1). Finally, it was verified that the Y_1 specific agonists [Leu^{31},Pro^{34}]NPY and [D-Trp^{32}]NPY have similar low affinity for the rat brain recombinant Y_2 receptor and the rat crypt cell PYY receptor, whereas the Y_4 specific agonist PP (Yan et al., 1996) has very low affinity, if any, for both receptors (Table 1). From these data, it could be concluded that the most selective receptor agonists currently available do not discriminate between the rat intestinal PYY receptor and the recombinant rat brain Y_2 receptor. We also investigated the effect of BIIE0246 which is a new specific Y_2 receptor antagonist (Dumont et al., 2000), on ^{125}I-PYY binding (Fig. 1). It appears that BIIE0246 competes with tracer

TABLE 1

Binding properties of PYY, NPY, PP, analogs, and the Y_2 receptor antagonist BIIE0246

K_i values and 95% confidence intervals were calculated from competition binding data (three to six experiments) using the GraphPad Prism software with a fit to a sigmoidal concentration-response curve.

Peptides	K_i	
	Rat Jejunal Crypt Cells	Recombinant Rat Y_2 Receptor
	nM	
p PYY	0.06 (0.03–0.10)	0.09 (0.07–0.12)
p $PYY_{3–36}$	0.26 (0.18–0.37)	0.12 (0.09–0.17)
N-α-Ac-$PYY_{22–36}$	0.54 (0.45–0.63)	1.66 (1.07–2.58)
N-α-Ac-$PYY_{25–36}$	0.21 (0.16–0.27)	0.77 (0.22–2.65)
h,r NPY	0.79 (0.39–1.58)	0.42 (0.28–0.63)
p $NPY_{3–36}$	1.04 (0.76–1.42)	0.84 (0.59–1.18)
h,r $NPY_{13–36}$	2.62 (1.08–6.32)	1.28 (0.84–1.94)
h,r [Leu^{31},Pro^{34}] NPY	393 (252–615)	>1,000
h,r [D-Trp^{32}] NPY	276 (97–787)	48.7 (17.9–132.1)
C2-NPY	0.07 (0.04–0.13)	0.15 (0.03–0.61)
r PP	>1,000	>1,000
h PP	>1,000	>1,000
BIIE0246	6.5 (2.3–18.9)	9.0 (6.7–12.2)

similarly in crypt cell and transfected CHO cell membranes (Fig. 1) with a K_i of 6.5 nM (95% confidence interval, 2.3–18.9) and 9 nM (6.7–12.2), respectively. In contrast, the Y_1 receptor antagonist 1229U91 (tested at concentrations up to 1 μM), also considered a Y_4 receptor agonist (Gehlert, 1998), failed to compete with ^{125}I-PYY for binding to membranes from crypt cells or transfected CHO cells (data not shown).

Further experiments investigated the action of BIIE0246 on the inhibition of cAMP production by PYY. As shown in Fig. 2, PYY inhibits cAMP production in intact crypt cells or transfected CHO cells with an EC_{50} value of 3.8 nM (95% confidence interval, 0.5–25.0) and 15.5 nM (6.9–31.2), respectively. Addition of the Y_2 receptor antagonist BIIE0246 at 1 μM completely abolished the inhibitory action of PYY in both crypt cells and transfected CHO cells (Fig. 2). Moreover, it was verified that increasing concentrations of BIIE0246 from 0.1 nM to 1 μM did antagonize the inhibitory effect of 0.1 μM PYY (Fig. 3) in a concentration-dependent manner, which represents 90% of the maximal inhibition that can be evoked by PYY (Fig. 2).

If the intestinal cryptic PYY receptor is a Y_2 receptor, as suggested by the data described above, then the Y_2 receptor antagonist BIIE0246 should antagonize the inhibitory effect of PYY on electrolyte secretion in rat small intestine. This was investigated here both in vitro and in vivo. First we examined the action of BIIE0246 on short-circuit current (Isc) in the Ussing chamber model. PYY (10 or 100 nM) was added to the serosal side of rat jejunum mounted in Ussing chamber after a 30-min basal state was reached (Isc = 70.6 ± 5.3 μA/cm^2; conductance = 24.3 ± 0.9 mS/cm^2, n = 20). Basal Isc dropped significantly ($p < 0.01$) in response to either concentration of PYY (Fig. 4) without significant change in tissue conductance (not shown). Maximal decrease of Isc in response to 10 nM PYY (ΔIsc = −26.4 ± 1.6 μA/cm^2, n = 4) or 100 nM PYY (ΔIsc = −36.7 ± 7.8 μA/cm^2, n = 4) was reached within 6 to 8 min when Isc slowly began to increase for another 10 to 15 min (Fig. 4, B and C). Addition of 1 μM BIIE0246 alone had no effect on Isc (Fig. 4A) or tissue con-

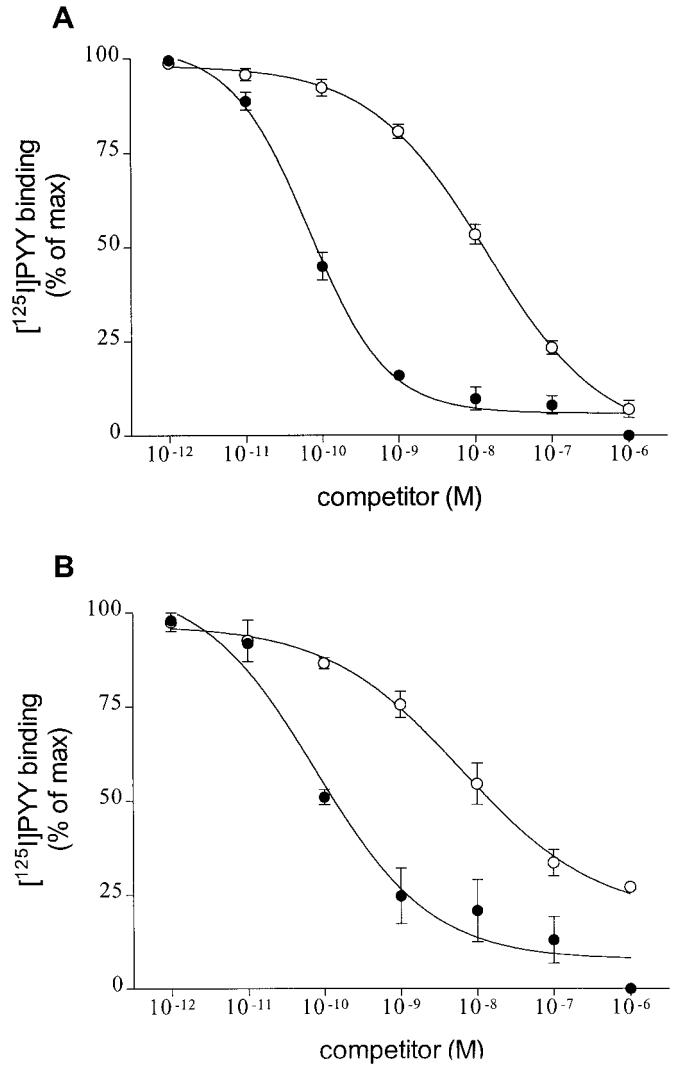

Fig. 1. Competition with ^{125}I-PYY for binding to membrane preparations of CHO cells stably transfected with rat hippocampal Y_2 receptor cDNA (A) and of rat jejunal crypt cells (B). The competitors were PYY (●) and the Y_2 receptor antagonist BIIE0246 (○). Data represent the mean ± S.E.M. of at least three determinations, each performed in triplicate.

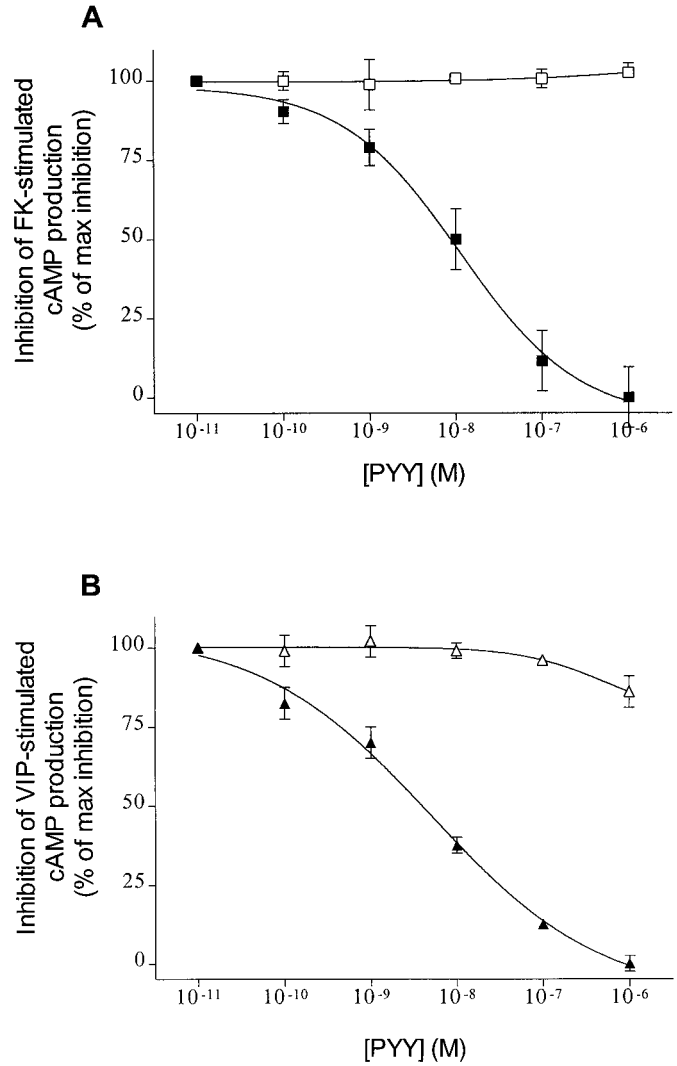

Fig. 2. Inhibition of stimulated cAMP production by increasing concentrations of PYY in the presence or absence of the Y_2 receptor antagonist BIIE0246. A, CHO cells stably transfected with rat hippocampal Y2 receptor cDNA. Cyclic AMP production was stimulated by 10^{-5} M forskolin (FK). ■, PYY alone; □, PYY + 10^{-6} M BIIE0246. B, rat jejunal crypts cells. Cyclic AMP production was stimulated by 10^{-7} M VIP. ▲, PYY alone; △, PYY + 10^{-6} M BIIE0246. Data represent the mean ± S.E.M. of at least three determinations, each performed in triplicate.

ductance (not shown). When BIIE0246 was added together with either 10 or 100 nM PYY, the maximal decrease of Isc in response to PYY was reduced significantly: $\Delta Isc = -6.8 \pm 1.9$ $\mu A/cm^2$ ($n = 5$, $p < 0.01$ versus PYY alone) and -17.9 ± 4.6 $\mu A/cm^2$ ($n = 4$, $p < 0.01$ versus PYY alone), respectively (Fig. 4, B and C). In addition, BIIE0246 also induced a rapid return to basal Isc value that was achieved in 8 to 10 min after maximal decrease [i.e., significantly shorter than with 10 or 100 nM PYY alone ($p < 0.05$)]. The action of BIIE0246 in Ussing chamber experiments led us to investigate its effect on net fluid secretion in the rat jejunum in vivo. For that purpose, we studied the action of BIIE0246 on the antisecretory effect of PYY on jejunal net water flux across rat jejunal ligatured loop, stimulated by VIP. In basal conditions, the jejunal mucosa demonstrated a large absorptive flux (Fig. 5): -39.8 ± 4.8 μl/cm/30 min, which was reverted to secretion: 36.8 ± 2.7 μl/cm/30 min ($P < 0.001$) by 100 μg/kg/h VIP. The secretory effect of VIP was inhibited by 300 pmol/kg.h PYY: 11.7 ± 3.0 μl/cm/30 min (Fig. 5, lane VIP + PYY). The effect of BIIE0246, was studied first on basal, then against PYY. We showed that 30 nmol/kg/h of BIIE0246 reversed by 100% the PYY inhibition of VIP-stimulated secretion ($P < 0.001$) (Fig. 5, lane VIP + PYY + BIIE0246). BIIE0246 alone did not change basal net water flux (Fig. 5, lane BIIE0246). Nor did it change the VIP-induced secretion (Fig. 5, lane VIP + BIIE0246).

The rat intestinal PYY-preferring receptor is expressed in crypt cells in which hydroelectrolytic secretion is believed to take place (Voisin et al., 1990), whereas a very low amount of

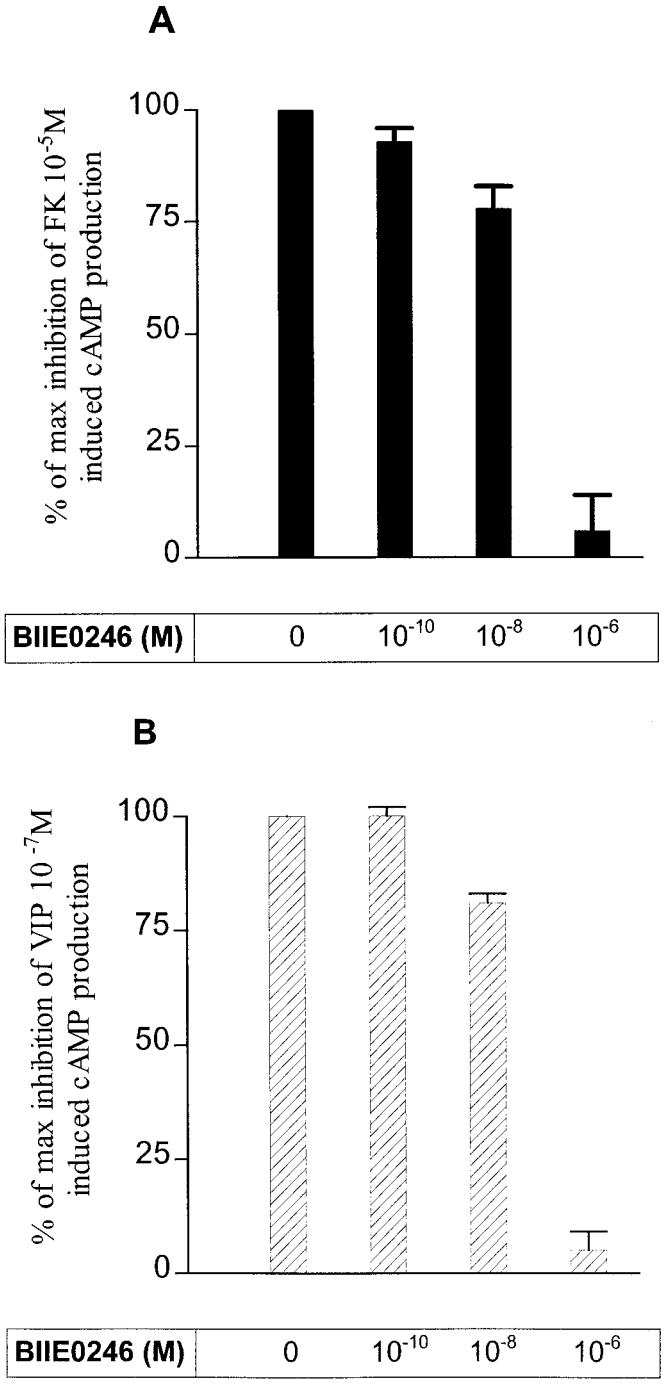

Fig. 3. Reversion of PYY-inhibited cAMP production by increasing concentrations of the Y_2 receptor antagonist BIIE0246. A, CHO cells stably transfected with rat hippocampal Y_2 receptor cDNA. Forskolin (FK; 10^{-5} M)-stimulated cyclic AMP production was inhibited by 10^{-7} M PYY. B, rat jejunal crypts cells. VIP (10^{-7} M)-stimulated cyclic AMP production was inhibited by 10^{-7} M PYY. Data represent the mean ± S.E.M. of three determinations, each performed in triplicate.

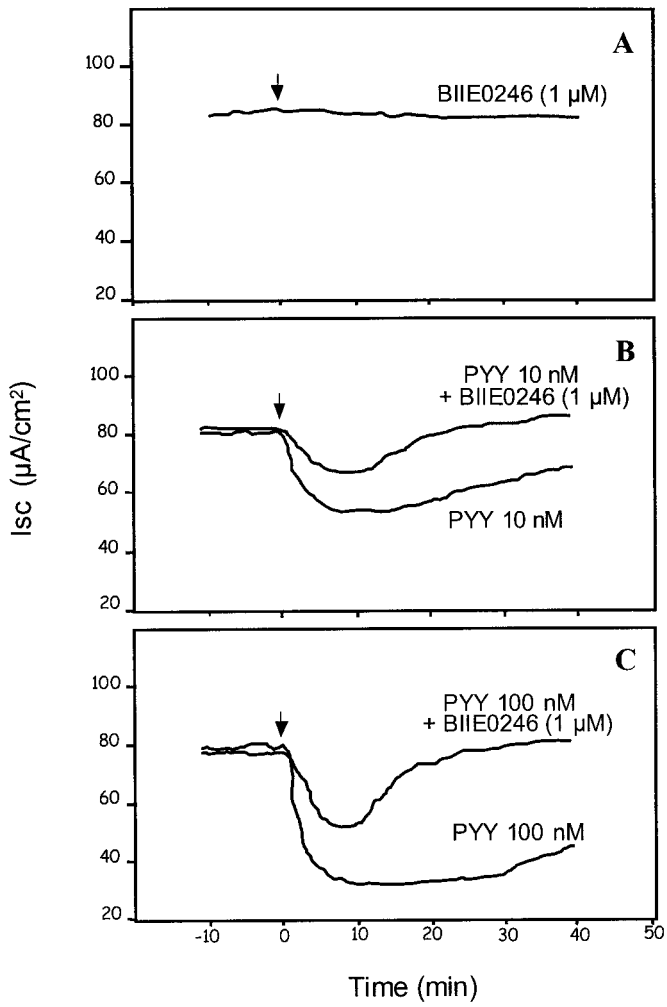

Fig. 4. Reduction of short-circuit current (Isc) across rat jejunum by PYY: effect of the Y_2 receptor antagonist BIIE0246. Compounds were added (arrow) to the serosal side of rat jejunum mounted in Ussing chamber after a 30-min basal state was reached. A, BIIE0246 (1 μM) alone. B, PYY (10 nM) in the absence or presence of BIIE0246 (1 μM). C, PYY (100 nM) in the absence or presence of BIIE0246 (1 μM). Typical experiments are shown. Four other experiments gave similar data.

PYY receptor, if any, is detected in villus cells (Voisin et al., 1990). However, previous RT-PCR studies using human Y_2 receptor primers previously identified transcripts in both crypt cells and villus cells, an observation that apparently disagreed with functional data (Goumain et al., 1998b). Therefore, we investigated here the expression of Y_2 receptor transcripts in isolated crypt cells and villus cells by quantitative RT-PCR using rat Y_2 receptor primers chosen in the recently available rat brain Y_2 receptor sequence (St. Pierre et al., 1998). A single amplicon of the expected size (442 bp) was observed with RNA extracted from rat crypt cells (Fig. 6A). Characterization of RT-PCR product by the restriction enzyme *Bgl*II yielded two fragments (Fig. 6B) of expected sizes (315 and 127 bp) strongly supporting that the amplification product corresponded to Y_2 receptor sequence. For evaluation of Y_2 receptor mRNA levels, quantitative RT-PCR of Y_2 receptor was carried out on rat jejunal crypt or villus cells RNA_T (total RNA) together with coamplification with 10^3 molecules of specific internal standard DNA (Fig. 6A). Two serial amplicons of the expected sizes were obtained (i.e., 442 bp for Y_2 receptor cDNA and 489 bp for internal standard cDNA). After RT-PCR using Y_2 receptor primers, a labeled band of 442 bp was observed in rat jejunal crypt cells, whereas no band could be detected in rat jejunal villus cells (Fig. 6A, lanes 2 and 4). The presence of PCR products for rat β-actin taken as a control for a housekeeping gene, was observed in all cell preparations (Fig. 6A, bottom). The evaluation of Y_2 receptor mRNA level in crypt cells using quantitative RT-PCR showed that 3×10^2 mRNA molecules/100 ng RNA_T were expressed in crypt cells, whereas no transcript could be detected in villus cells under the same experimental conditions. These data supported that Y_2 receptor mRNA was mainly expressed in rat jejunal crypt cells with no significant expression in rat jejunal villus cells. This distribution is identical to the distribution of ^{125}I-PYY binding in isolated epithelial cells from rat crypt and villus (Voisin et al., 1990). Because no ^{125}I-PYY binding or inhibition of cAMP production by PYY could be observed in epithelial cells isolated from rat colon (Laburthe et al., 1986; Servin et al., 1989), similar RT-PCR experiments were also carried out on RNA_T isolated from rat colonic epithelial cells. No Y_2 receptor transcript could be detected either in colon epithelium (Fig. 6), further supporting the validity of our data.

The RT-PCR experiments described above identified the prevalent expression of Y_2 receptor transcripts in crypt cells but did not strictly establish the identity between the Y_2 receptor cloned in rat brain (St. Pierre et al., 1998) and a Y_2 receptor present in rat crypt cells. Indeed, the primers used encompassed only a portion of the rat brain Y_2 cDNA receptor sequence from 350 to 791 corresponding to residues 118 to 263 in the receptor protein sequence (381 amino acids, full length). Therefore, we performed additional RT-PCR experiments on RNA_T extracted from rat crypt cells using several other couples of primers including a couple of primers that fully overlap the Y_2 receptor sequence determined in rat brain (see *Experimental Procedures*). Single PCR products of the expected sizes were obtained for every oligonucleotide combinations (Fig. 7). Each of these PCR products was subcloned and sequenced, including the full-length sequence expected with the combination of oligo 1 and oligo 5 (see Fig. 7). The obtained full-length nucleotide sequence for the rat intestinal Y_2 receptor and its deduced amino acid sequence are shown on Fig. 8. Comparison of the nucleotide sequence of this rat intestinal Y_2 receptor with that of the cloned rat brain Y_2 receptor (St. Pierre et al., 1998) revealed 100% identity. The same held true for other PCR products obtained, which also exhibited 100% identity with the corre-

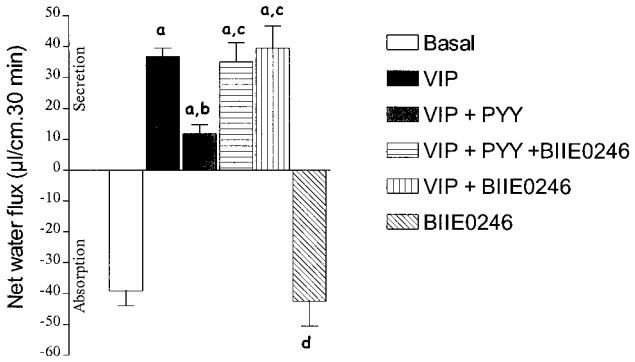

Fig. 5. Effect of the Y_2 receptor antagonist BIIE0246 on the antisecretory effect of peptide YY on VIP-stimulated jejunal net water flux in vivo in rats. The effect of Y_2 receptor antagonist (BIIE0246, 30 nmol/kg/h) is shown by comparing the net water flux after VIP (100 μg/kg/h) + PYY (300 pmol/kg/h) with antagonist to the control group VIP + PYY without antagonist. Data represent means ± S.E.M. in groups of six to eight rats. a, $P < 0.001$ versus basal; b, $P < 0.001$ versus VIP; c, not significant versus VIP; d, not significant versus basal.

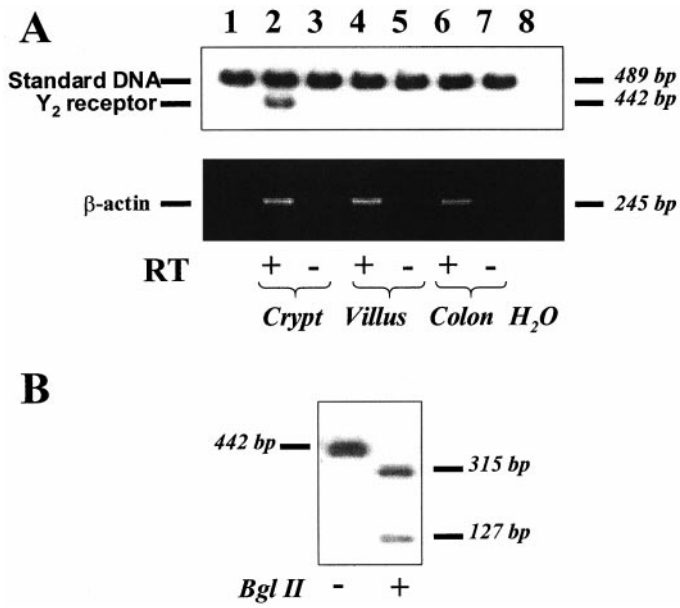

Fig. 6. Quantitative RT-PCR of Y_2 receptor gene transcripts in rat epithelial intestinal cells. Total RNA from jejunal crypt cells, jejunal villus cells, and colonic cells were studied by RT-PCR. The cDNA products obtained by RT-PCR were resolved by 4% polyacrylamide gels and analyzed by autoradiography. A, top, coamplification of specific internal DNA standard (103 molecules) with reverse-transcribed product from rat crypt cells (lane 2), villus cells (lane 4), or colonic cells (lane 6) target RNA_T. Two serial bands of the expected size (upper, 489 bp for the internal standard; lower, 442 bp for the Y_2 receptor) were observed. Control assays were performed in the presence of internal DNA standard and in the absence of RNA_T (lane 1), in the presence of RNA_T and in the absence of reverse transcriptase (lanes 3, 5, and 7) or with water instead of cDNA preparations (lane 8). Bottom, the presence of PCR products (expected size, 245 bp) for β-actin was taken as control for a house-keeping gene. B, specific characterization of Y_2 receptor amplicon from jejunal crypt cells by *Bgl*II restriction enzyme digestion. The expected band sizes for Y_2 receptor fragment were 442 bp (uncut control) and 315 + 127 bp (*Bgl*II digestion).

sponding sequences of the rat brain Y_2 cDNA. Because several PCR products were cloned and sequenced, it can be concluded that a peripheral Y_2 receptor identical to the brain Y_2 receptor does exist in the rat intestinal epithelial crypt cells.

To extend our observations to human intestine, we cloned by PCR a human Y_2 receptor from a human small intestinal cDNA library using brain Y_2 receptor oligonucleotides. The PCR products obtained (Fig. 9A) fully overlapped the Y_2 receptor sequence. Sequencing the two PCR products (data not shown) revealed 100% identity between the human Y_2 receptors in small intestine and brain (Gerald et al., 1995; Rose et al., 1995). These data indicated that a Y_2 receptor does exist in human small intestine. Binding experiments carried out with the human Y_2 receptor transiently expressed in CHO cells (Fig. 9B) led us to observe the following order of affinity for PYY, NPY, and two analogs: hPYY [K_i = 1.2 nM (0.9–1.4)] > hNPY [K_i = 6.7 nM (3.4–13.0)] > PYY_{3-36} [K_i = 9.3 nM (7.2–12.0)] > *N*-α-Ac-PYY_{22-36} [K_i = 28.2 nM (18.2–44.0)]. These data indicated that the human Y_2 receptor expressed in CHO cells exhibits a PYY-preferring profile like the rat Y_2 receptor and recognizes the short PYY_{22-36} fragment with a significant affinity.

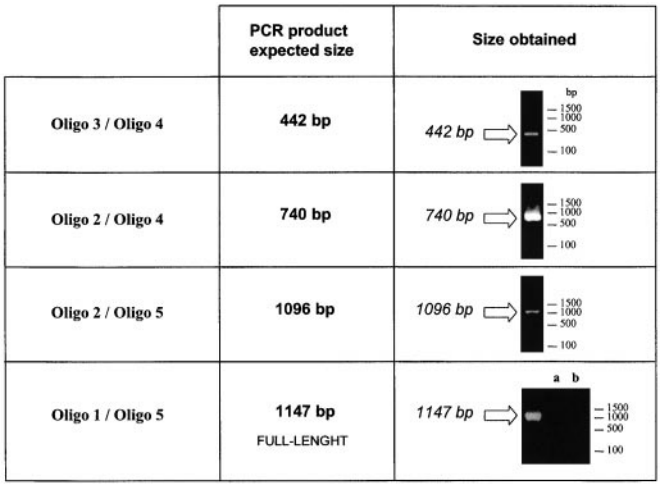

Fig. 7. Rat Y_2 receptor gene product amplification in rat epithelial intestinal cells using several combinations of oligonucleotides. The oligonucleotides used (see *Experimental Procedures*) encompass different portion of the Y_2 receptor including a full-length receptor (Oligo 1/Oligo 5 combination). Lane a, PCR was conducted without retrotranscription step; lane b, negative control with cDNA being replaced with water. All PCR products were subcloned and sequenced.

Discussion

A very early description of a receptor for PYY and NPY was the characterization of specific binding of ^{125}I-PYY to rat intestinal epithelial cells (Laburthe et al., 1986). The term of PYY-preferring receptor was used at that time because PYY was 5 to 10 times more potent than NPY (Laburthe et al., 1986; Laburthe, 1991). Further studies on PYY-induced inhibition of cAMP production in intestinal cells (Servin et al., 1989) and PYY-induced inhibition of intestinal water and electrolytes secretion in various in vitro and in vivo models (Cox et al., 1988; Cox and Cuthbert, 1990; Balasubramaniam et al., 1993, 2000) confirmed the PYY preference. This property has recently been claimed as being common to receptors in gastrointestinal epithelia (Holliday et al., 2000). However, it could not be considered the hallmark of an additional Y receptor subtype, because the preference is of small magnitude and a PYY-preferring receptor has not been cloned. In this context, the present study provides strong arguments supporting that the historical PYY-preferring receptor in rat intestinal epithelium is a peripheral Y_2 receptor.

Although Y_2 receptor was cloned in 1995 in humans (Gerald et al., 1995; Rose et al., 1995), no Y_2 receptor cDNA had been isolated in rats until recently (St. Pierre et al., 1998). Because there are important pharmacological differences between species for Y receptors (Gehlert, 1998), the pharmacological profile of the rat intestinal PYY receptor could not be compared directly with that of the Y_2 receptor from humans. In this work, a CHO cell clone stably expressing the rat hippocampal Y_2 receptor was developed. It displayed binding parameters similar to that of the PYY receptor in rat crypt cells in terms of binding capacity and dissociation constant for PYY, allowing direct comparison of receptors from the two sources. First, it could be noted that the PYY preference was also observed for the recombinant rat Y_2 receptor expressed

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ATG GGC CCA TTA GGT GCA GAG GCA GAT GAG AAT CAA ACT GTA GAA GTG AAA GTG GAA CTC TAT GGG TCG GGG CCC ACC ACT CCT AGA GGT    90
 M   G   P   L   G   A   E   A   D   E   N   Q   T   V   E   V   K   V   E   L   Y   G   S   G   P   T   T   P   R   G     30

GAG TTG CCC CCT GAT CCA GAG CCG GAG CTC ATA GAC AGC ACC AAA CTG GTT GAG GTG CAG GTG GTC CTT ATA CTG GCC TAT TGT TCC ATC    180
 E   L   P   P   D   P   E   P   E   L   I   D   S   T   K   L   V   E   V   Q   V   V   L   I   L   A   Y   C   S   I     60
                                                                                 I

ATC TTG CTG GGC GTA GTT GGC AAC TCT CTG GTA ATC CAT GTG GTG ATC AAA TTC AAG AGC ATG CGC ACA GTA ACC AAC TTT TTT ATT GCC    270
 I   L   L   G   V   V   G   N   S   L   V   I   H   V   V   I   K   F   K   S   M   R   T   V   T   N   F   F   I   A     90
                                                                                                     II

AAC CTG GCT GTG GCG GAT CTT TTG GTG AAC ACC CTG TGC CTG CCA TTC ACT CTT ACC TAT ACC TTG ATG GGG GAG TGG AAA ATG GGT CCA    360
 N   L   A   V   A   D   L   L   V   N   T   L   C   L   P   F   T   L   T   Y   T   L   M   G   E   W   K   M   G   P    120

GTT TTG TGC CAT TTG GTG CCC TAT GCC CAG GGT CTG GCA GTA CAA GTG TCC ACA ATA ACT TTG ACA GTC ATT GCT TTG GAC CGA CAT CGT    450
 V   L   C   H   L   V   P   Y   A   Q   G   L   A   V   Q   V   S   T   I   T   L   T   V   I   A   L   D   R   H   R    150
                 III

TGC ATT GTC TAC CAC CTG GAG AGC AAG ATC TCC AAG CAA ATC AGC TTC CTG ATT ATT GGC CTG GCG TGG GGT GTC AGC GCT CTG CTG GCA    540
 C   I   V   Y   H   L   E   S   K   I   S   K   Q   I   S   F   L   I   I   G   L   A   W   G   V   S   A   L   L   A    180
                                                 IV

AGT CCC CTT GCC ATC TTC CGG GAG TAC TCA CTG ATT GAG ATT ATT CCT GAC TTT GAG ATT GTA GCC TGT ACT GAG AAA TGG CCC GGG GAG    630
 S   P   L   A   I   F   R   E   Y   S   L   I   E   I   I   P   D   F   E   I   V   A   C   T   E   K   W   P   G   E    210

GAG AAG AGT GTG TAC GGT ACA GTC TAC AGC CTT TCC ACC CTG CTA ATC CTC TAC GTT TTG CCT CTG GGC ATC ATA TCT TTC TCC TAC ACC    720
 E   K   S   V   Y   G   T   V   Y   S   L   S   T   L   L   I   L   Y   V   L   P   L   G   I   I   S   F   S   Y   T    240
                                                     V

CGG ATC TGG AGT AAG CTA AAG AAC CAC GTT AGT CCT GGA GCT GCA AGT GAC CAT TAC CAT CAG CGA AGG CAC AAA ACG ACC AAA ATG CTC    810
 R   I   W   S   K   L   K   N   H   V   S   P   G   A   A   S   D   H   Y   H   Q   R   R   H   K   T   T   K   M   L    270
                                                                                                             VI

GTG TGC GTG GTA GTG GTG TTT GCA GTC AGC TGG CTG CCC CTC CAT GCC TTC CAA CTT GCT GTG GAC ATC GAC AGC CAT GTC CTG GAC CTG    900
 V   C   V   V   V   V   F   A   V   S   W   L   P   L   H   A   F   Q   L   A   V   D   I   D   S   H   V   L   D   L    300

AAG GAG TAC AAA CTC ATC TTC ACC GTG TTC CAC ATT ATT GCG ATG TGC TCC ACC TTC GCC AAC CCC CTT CTC TAT GGC TGG ATG AAC AGC    990
 K   E   Y   K   L   I   F   T   V   F   H   I   I   A   M   C   S   T   F   A   N   P   L   L   Y   G   W   M   N   S    330
                 VII

AAC TAC AGA AAA GCT TTC CTC TCA GCC TTC CGC TGT GAG CAG AGG TTG GAT GCC ATT CAC TCG GAG GTG TCC ATG ACC TTC AAG GCT AAA   1080
 N   Y   R   K   A   F   L   S   A   F   R   C   E   Q   R   L   D   A   I   H   S   E   V   S   M   T   F   K   A   K    360

AAG AAC CTG GAA GTC AAA AAG AAC AAT GGC CTC ACT GAC TCT TTT TCA GAG GCC ACC AAC GTG TAA G                               1147
 K   N   L   E   V   K   K   N   N   G   L   T   D   S   F   S   E   A   T   N   V   *                                 382
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Fig. 8. Full-length rat jejunal crypt Y_2 receptor cDNA. Putative seven transmembrane domains are underlined. The full-length rat intestinal Y_2 receptor cDNA was submitted to GenBank (accession number AY004257).

in CHO cells. Moreover, the typical selective Y_2 agonists (Michel et al., 1998) $PYY_{3–36}$, $PYY_{13–36}$, $NPY_{3–36}$, and $NPY_{13–36}$ or the very short PYY analogs acting at the intestinal PYY receptor, *N*-α-$AcPYY_{22–36}$ and *N*-α-$AcPYY_{25–36}$, could not discriminate between the two receptors (see Table 1). Finally, the Y_2 receptor antagonist BIIE0246 displayed identical affinity for the rat intestinal PYY receptor and the recombinant rat Y_2 receptor. This first set of experiments supported the idea that the intestinal PYY-preferring receptor is indistinguishable from a Y_2 receptor at the binding level.

The development of the potent and selective Y_2 receptor antagonist BIIE0246 (Dumont et al., 2000) provided the opportunity to further characterize PYY receptor-mediated proximal and distal responses in small intestine. We showed in this work that BIIE0246 blocked PYY-induced inhibition of cAMP production in isolated jejunal crypt cells, PYY-induced decrease of short-circuit current in rat jejunum mucosa mounted in Ussing chamber, and PYY-induced inhibition of fluid secretion in rat jejunum in vivo. This is in line with the high-affinity binding of BIIE0246 to PYY receptor in intestinal crypt cells and supports that an epithelial Y_2 receptor mediates, at least in part, the antisecretory action of PYY in small intestine (Fu-Cheng et al., 1999). The Y_2 receptor antagonist BIIE0246 completely blocked the long-term (>10 min) effect of PYY on short-circuit current in the Ussing chamber assay but only partially blocked the short-term effect (<10 min) of the peptide (see Fig. 4). This is in contrast to the total inhibition seen in other assays including the cAMP assay in vitro (see Fig. 2) and the intestinal secretion assay in vivo (see Fig. 5). Although it may be suggested that the data obtained in the Ussing chamber assay did not support the idea of the sole mediation of PYY effect via a Y_2 receptor, the in vivo assay clearly indicated a Y_2 receptor-mediated antisecretory action of PYY in rat small intestine. Because the cAMP assay in vitro and the secretion assay in vivo were carried out over 45- and 30-min periods, respectively, it can be suggested that the partial effect of BIIE0246 over < 10 min period in the Ussing chamber assay is related to differences in the time course of action of BIIE0246 and PYY that were no longer observed over long incubation periods. Previous studies have shown that PYY and NPY act upon epithelia of the rat small intestine causing a reduction in short-circuit current that is not sensitive to tetrodotoxin (TTX) and is thereby not neurally mediated (Cox et al., 1988). However, this has not been confirmed by Fu-Cheng et al. (1999), who claimed that TTX and several other neural antagonists did reduce PYY effect in the rat jejunum in vitro. Furthermore, other in vivo studies indicated that the PYY-induced inhibition of rat small intestinal fluid secretion can be abolished, at least in part, by TTX or hexamethonium, suggesting neuronal mediation (Souli et al., 1997). The respective contribution of epithelium and neurons in the inhibition of intestinal secretion by PYY certainly deserves further investigation, but our data showing that BIIE0246 completely blocked the action of PYY in isolated crypt cells, in jejunal mucosa in vitro, and in rat jejunum in vivo support the idea that all the actions of PYY on inhibition of fluid secretion in rat small intestine are mediated by Y_2 receptors. In this context, it is worth pointing out that neurally mediated action of PYY on inhibition of fluid secretion may be prevalent in humans (Roze et al., 1997). While this work was in progress, we were very interested in the inactivation of Y_2 receptor in mice (Naveilhan et al., 1999) because availability of $Y_2^{-/-}$ mice could have been a clue to our present studies. However, as we were testing $Y_2^{-/-}$ mice for expression of PYY-preferring receptor, we observed that intestinal epithelial cells from normal mice did not exhibit any ^{125}I-PYY binding (unpublished data). This was in agreement with the absence of effect of PYY on short-circuit current (Isc) in jejunal segment of BALB/c mice mounted in Ussing chamber (authors' unpublished observations). This observation further highlights the issue of species difference in the expression of receptors in the digestive tract and indicates that $Y_2^{-/-}$ mice could not help in solving the problem raised by the present study.

We reasoned that if Y_2 receptor were responsible for PYY binding in rat intestinal epithelium, we should find a good correlation between the expression of Y_2 receptor mRNA and functional studies of PYY binding or PYY-induced inhibition

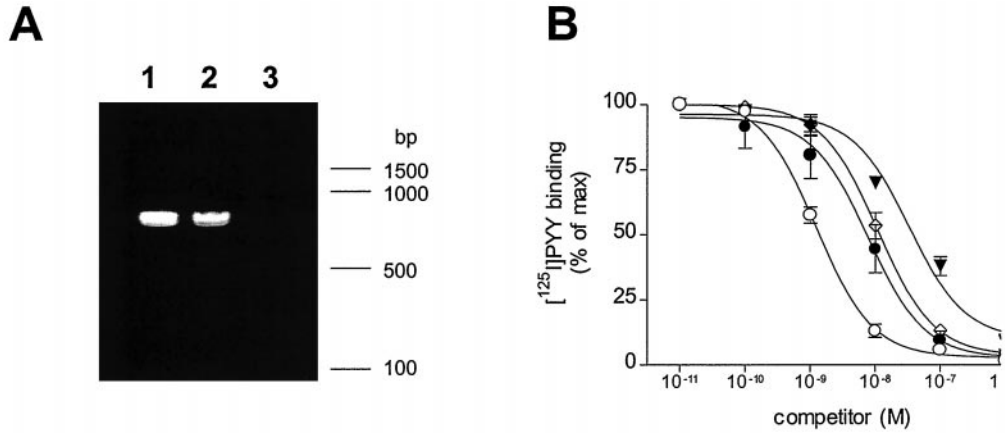

Fig. 9. Human intestinal Y_2 receptor cloning and expression in CHO cells. A, PCR was carried out using human small intestinal cDNAs (QUICK-clone cDNA from CLONTECH Laboratories) and two combinations of oligonucleotides. The oligonucleotides used (see *Experimental Procedures*) encompass two portions of the human Y_2 receptor including a 442-bp overlapping sequence. Lane 1, PCR was conducted with oligo A/oligo C combination; lane 2, PCR was conducted with oligo B/oligo D combination; lane 3, negative control, cDNA replaced with water. The two PCR products (791 bp and 797 bp, respectively) were subcloned and sequenced. B, peptide specificity of human Y_2 receptor expressed in CHO cells. Membranes were incubated with 0.05 nM ^{125}I-PYY and various concentrations of unlabeled hPYY (○), hNPY (●), $hPYY_{3–36}$ (◇), and *N*-α-Ac-$PYY_{22–36}$ (▼). Results are the means ± S.E. from five experiments.

of cAMP production in intestinal epithelial cells. However, previous studies using RT-PCR identified Y_2 transcripts in crypt cells and villus cells from rat small intestine and in rat colon epithelial cells (Goumain et al., 1998b), whereas PYY binding (Laburthe et al., 1986; Voisin et al., 1990) and PYY-induced inhibition of cAMP production (Servin et al., 1989; Voisin et al., 1990) could be only observed in small intestinal crypt cells. To document this issue, we developed here quantitative RT-PCR for Y_2 receptor mRNA. The data clearly demonstrated that under our experimental conditions in which 3×10^2 Y_2 receptor mRNA molecules/100 ng RNA_T were measured in crypt cells, no transcript could be detected in villus cells or colon epithelial cells. Therefore, there is an excellent correlation between the expression of Y_2 receptor mRNA and ^{125}I-PYY binding in the different epithelial cell populations isolated from rat small intestine and colon. Previous amplification conditions (35 cycles using 2.5 μg RNA_T) most probably detected traces of Y_2 receptor mRNA in villus cells or colon epithelial cells (Goumain et al., 1998b), whereas the present conditions (25 cycles using 0.1 μg RNA_T) did not.

To provide definitive evidence of the existence of Y_2 receptor in rat intestinal crypt cells, full-length Y_2 receptor cDNAs were cloned by RT-PCR from RNA_T isolated from these cells. The four clones isolated in separate experiments exhibited 100% identity with the rat hippocampal Y_2 receptor cDNA (St. Pierre et al., 1998). Neither fragment of Y_2 receptors that were amplified using several couples of primers (see Fig. 7) exhibited difference with the rat hippocampal Y_2 receptor sequence. Therefore, it could be concluded that Y_2 receptor does exist in rat crypt cells with no evidence for variants in our experiments. This rat Y_2 receptor exhibits PYY preference in crypt cells or after transfection in CHO cells, whereas previously cloned Y_2 receptors from human nervous tissues exhibited very small PYY preference when transfected in COS cells (Gerald et al., 1995; Rose et al., 1995). The reasons for this remain unclear. This may be tentatively ascribed to species differences in the properties of Y_2 receptor and/or to differences in G protein composition or availability in the gut versus the brain. It is clear from this study that human small intestine is equipped with a Y_2 receptor that is 100% homologous to the human brain Y_2 receptor and exhibits a PYY-preferring profile when expressed in CHO cells (see Fig. 9). The concept of PYY preference (Laburthe et al., 1986; Laburthe, 1991) can probably be extended to Y_2 and/or Y_1 receptors in many tissues including human adipose tissue (Castan et al., 1993), mouse kidney (Voisin et al., 1993), and human (Cox and Tough, 1995), rat, and mouse (Holliday et al., 2000) gastrointestinal epithelia. It may be functionally relevant to peripheral targets that have access to both NPY released from nerve endings and blood-borne PYY. Finally, it is worth pointing out that although the present study demonstrates that the PYY-preferring receptor in small intestine is a Y_2 receptor, we cannot strictly exclude that one or more PYY-preferring but still-unidentified receptors exist in mammals.

In conclusion, the present work demonstrates that the so-called PYY-preferring receptor mediating inhibition of small intestinal secretion by PYY or NPY is a peripheral Y_2 receptor. This demonstration puts an end to previous suggestions regarding the existence of a PYY-preferring receptor subtype in small intestine, which would represent an additional Y receptor. It also indicates that the potent and long-lasting proabsorptive and antisecretory PYY_{22-36} analogs recently developed for intestinal PYY receptor (Balasubramaniam et al., 2000) may have broader value as Y_2 receptor agonists in various tissues. Whether other putative NPY receptor subtypes, such as Y_3 receptor (Michel et al., 1998), grant an individual receptor status remains to be determined.

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References

Balasubramaniam A and Sheriff S (1990) Neuropeptide Y_{18-36} is a competitive antagonist of neuropeptide Y in rat cardiac ventricular membranes. *J Biol Chem* **265:**14724–14727.

Balasubramaniam A, Cox HM, Voisin T, Laburthe M, Stein M and Fischer JE (1993) Structure-activity studies of peptide YY_{22-36}: *N*-Alpha-Ac-[Phe^{27}]PYY_{22-36}, a potent antisecretory peptide in rat jejunum. *Peptides* **14:**1011–1016.

Balasubramaniam A, Tao Z, Zhai W, Stein M, Sheriff S, Chance WT, Fischer JE, Eden PE, Taylor JE, Liu CD, et al. (2000) Structure-activity studies including a Psi(CH_2-NH) scan of peptide YY (PYY) active site, PYY_{22-36}, for interaction with rat intestinal PYY receptors: development of analogues with potent in vivo activity in the intestine. *J Med Chem* **43:**3420–3427.

Bard JA, Walker MW, Branchek TA and Weinshank RL (1995) Cloning and functional expression of a human Y_4 subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. *J Biol Chem* **270:**26762–26765.

Blomqvist AG and Herzog H (1997) Y-receptor subtypes-How many more ? *Trends Neurosci* **20:**294–298.

Burkhoff A, Linemeyer DL and Salon JA (1998) Distribution of a novel hypothalamic neuropeptide Y receptor gene and it's [sic] absence in rat. *Brain Res Mol Brain Res* **53:**311–316.

Castan I, Valet P, Larrouy D, Voisin T, Remaury A, Daviaud D, Laburthe M and Lafontan M (1993) Distribution of PYY receptors in human fat cells: an antilipolytic system alongside the α_2-adrenergic system. *Am J Physiol* **265:**E74–E80.

Cerda-Reverter JM and Larhammar D (2000) Neuropeptide Y family of peptides: structure, anatomical expression, function, and molecular evolution. *Biochem Cell Biol* **78:**371–392.

Chariot J, Tsocas A, Souli A, Presset O and Roze C (2000) Neural mechanism of the antisecretory effect of peptide YY in the rat colon in vivo. *Peptides* **21:**59–63.

Cox HM, Cuthbert AW, Hakanson R and Wahlestedt C (1988) The effect of neuropeptide Y and peptide YY on electrogenic ion transport in rat intestinal epithelia. *J Physiol (Lond)* **398:**65–80.

Cox HM and Cuthbert AW (1990) The effects of neuropeptide Y and its fragments upon basal and electrically stimulated ion secretion in rat jejunum mucosa. *Br J Pharmacol* **101:**247–252.

Cox HM and Tough IR (1995) Functional characterization of receptors with affinity for PYY, NPY, [Leu31,Pro34]NPY and PP in a human colonic epithelial cell line. *Br J Pharmacol* **116:**2673–2678.

Cox HM (1998) Peptidergic regulation of intestinal ion transport. A major role for neuropeptide Y and the pancreatic polypeptides. *Digestion* **59:**395–399.

Doods H, Gaida W, Wieland HA, Dollinger H, Schnorrenberg G, Esser F, Engel W, Eberlein W and Rudolf K (1999) BIIE0246: a selective and high affinity neuropeptide Y Y_2 receptor antagonist. *Eur J Pharmacol* **384:**R3–R5.

Dumont Y, Cadieux A, Doods H, Pheng LH, Abounader R, Hamel E, Jacques D, Regoli D and Quirion R (2000) BIIE0246, a potent and highly selective non-peptide neuropeptide Y Y_2 receptor antagonist. *Br J Pharmacol* **129:**1075–1088.

Eto B, Boisset M, Anini Y, Voisin T and Desjeux JF (1997) Comparison of the antisecretory effect of endogenous forms of peptide YY on fed and fasted rat jejunum. *Peptides* **18:**1249–1255.

Fu-Cheng X, Souli A, Chariot J and Roze C (1999) Antisecretory effect of peptide YY through neural receptors in the rat jejunum in vitro. *Peptides* **20:**987–993.

Gehlert DR, Beavers LS, Johnson D, Gackenheimer SL, Schober DA and Gadski RA (1996) Expression cloning of a human brain neuropeptide Y Y_2 receptor. *Mol Pharmacol* **49:**224–228.

Gehlert DR (1998) Multiple receptors for the pancreatic polypeptide (PP-fold) family: physiological implications. *Proc Soc Exp Biol Med* **218:**7–22.

Gerald C, Walker MW, Vaysse PJ, He C, Branchek TA and Weinshank RL (1995) Expression cloning and pharmacological characterization of a human hippocampal neuropeptide y/peptide YY Y_2 receptor subtype. *J Biol Chem* **270:**26758–26761.

Goumain M, Voisin T, Lorinet AM, Balasubramaniam A and Laburthe M (1998a) Pharmacological profile of the rat intestinal crypt peptide YY receptor *vs.* the recombinant rat Y_5 receptor. *Eur J Pharmacol* **362:**245–249.

Goumain M, Voisin T, Lorinet AM and Laburthe M (1998b) Identification and distribution of mRNA encoding the Y_1, Y_2, Y_4, and Y_5 receptors for peptides of the PP-fold family in the rat intestine and colon. *Biochem Biophys Res Commun* **247:**52–56.

Holliday ND, Pollock EL, Tough IR and Cox HM (2000) PYY preference is a common characteristic of neuropeptide Y receptors expressed in human, rat, and mouse gastrointestinal epithelia. *Can J Physiol Pharmacol* **78:**126–133.

Ingenhoven N and Beck-Sickinger AG (1999) Molecular characterization of the ligand-receptor interaction of neuropeptide Y. *Curr Med Chem* **6:**1055–1066.

Ingenhoven N, Eckard CP, Gehlert DR and Beck-Sickinger AG (1999) Molecular

characterization of the human neuropeptide Y Y_2-receptor. *Biochemistry* **38:**6897–6902.
Laburthe M, Amiranoff B and Boissard C (1982) Alpha-adrenergic inhibition of cyclic AMP accumulation in epithelial cells isolated from rat small intestine. *Biochim Biophys Acta* **721:**101–108.
Laburthe M, Chenut B, Rouyer-Fessard C, Tatemoto K, Couvineau A, Servin A and Amiranoff B (1986) Interaction of peptide YY with rat intestinal epithelial plasma membranes: binding of the radioiodinated peptide. *Endocrinology* **118:**1910–1917.
Laburthe M (1991) Peptide YY and neuropeptide Y in the gut: availability, biological actions, and receptors. *Trends Endocrinol Metab* **1:**168–147.
Laburthe M, Couvineau A and Voisin T (1999) Receptors for peptides of the VIP/PACAP and PYY/NPY/PP families, in *Gastrointestinal Endocrinology* (Greeley GH ed) pp 125–157, Humana Press, Totowa, NJ.
Lundell I, Blomqvist AG, Berglund MM, Schober DA, Johnson D, Statnick MA, Gadski RA, Gehlert DR and Larhammar D (1995) Cloning of a human receptor of the NPY family with high affinity for pancreatic polypeptide and peptide YY. *J Biol Chem* **270:**29123–29128.
Lundell I, Statnick MA, Johnson D, Schober DA, Starback P, Gehlert DR and Larhammar D (1996) The cloned rat pancreatic polypeptide receptor exhibits profound differences to the orthologous receptor. *Proc Natl Acad Sci USA* **93:**5111–5115.
Michel MC, Beck-Sickinger A, Cox H, Doods HN, Herzog H, Larhammar D, Quirion R, Schwartz T and Westfall T (1998) XVI. International union of pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol Rev* **50:**143–150.
Naveilhan P, Hassani H, Canals JM, Ekstrand AJ, Larefalk A, Chhajlani V, Arenas E, Gedda K, Svensson L, Thoren P, et al. (1999) Normal feeding behavior, body weight and leptin response require the neuropeptide Y Y_2 receptor. *Nat Med* **5:**1188–1193.
Playford RJ, Cox HM. Peptide YY and neuropeptide Y: two peptides intimately involved in electrolyte homeostasis. *Trends Pharmacol Sci* 1996;17:436–438.
Rose PM, Fernandes P, Lynch JS, Frazier ST, Fisher SM, Kodukula K, Kienzle B and Seethala R (1995) Cloning and functional expression of a cDNA encoding a human type 2 neuropeptide Y receptor [published erratum appears in *J Biol Chem* 1995;**270:**29038]. *J Biol Chem* **270:**22661–22664.
Rouet-Benzineb P, Buhler JM, Dreyfus P, Delcourt A, Dorent R, Perennec J, Crozatier B, Harf A and Lafuma C (1999) Altered balance between matrix gelatinases (MMP-2 and MMP-9) and their tissue inhibitors in human dilated cardiomyopathy: potential role of MMP-9 in myosin-heavy chain degradation. *Eur J Heart Fail* **1:**337–352.
Roze C, Molis C, Xiaomei FC, Ropert A, Geneve J and Galmiche JP (1997) Peptide YY inhibition of prostaglandin-induced intestinal secretion is haloperidol-sensitive in humans. *Gastroenterology* **112:**1520–1528.
Servin AL, Rouyer-Fessard C, Balasubramaniam A, Saint PS and Laburthe M (1989) Peptide-YY and neuropeptide-Y inhibit vasoactive intestinal peptide-stimulated adenosine 3′,5′-monophosphate production in rat small intestine: structural requirements of peptides for interacting with peptide-YY-preferring receptors. *Endocrinology* **124:**692–700.
Souli A, Chariot J, Presset O, Tsocas A and Roze C (1997) Neural modulation of the antisecretory effect of peptide YY in the rat jejunum. *Eur J Pharmacol* **333:**87–92.
St. Pierre JA, Dumont Y, Nouel D, Herzog H, Hamel E and Quirion R (1998) Preferential expression of the neuropeptide Y Y_1 over the Y_2 receptor subtype in cultured hippocampal neurons and cloning of the rat Y_2 receptor. *Br J Pharmacol* **123:**183–194.
Voisin T, Rouyer-Fessard C and Laburthe M (1990) Distribution of common peptide YY-neuropeptide Y receptor along rat intestinal villus-crypt axis. *Am J Physiol* **258:**G753–G759.
Voisin T, Couvineau A, Rouyer-Fessard C and Laburthe M (1991) Solubilization and hydrodynamic properties of active peptide YY receptor from rat jejunal crypts: characterization as a Mr 44,000 glycoprotein. *J Biol Chem* **266:**10762–10767.
Voisin T, Bens M, Cluzeaud F, Vandewalle A and Laburthe M (1993) Peptide YY receptors in the proximal tubule PKSV-PCT cell line derived from transgenic mice. Relation with cell growth. *J Biol Chem* **268:**20547–20554.
Voisin T, Goumain M, Lorinet AM, Maoret JJ and Laburthe M (2000) Functional and molecular properties of the human recombinant Y_4 receptor: resistance to agonist-promoted desensitization. *J Pharmacol Exp Ther* **292:**638–646.
Weinberg DH, Sirinathsinghji DJ, Tan CP, Shiao LL, Morin N, Rigby MR, Heavens RH, Rapoport DR, Bayne ML, Cascieri MA, et al. (1996) Cloning and expression of a novel neuropeptide Y receptor. *J Biol Chem* **271:**16435–16438.
Yan H, Yang J, Marasco J, Yamaguchi K, Brenner S, Collins F and Karbon W (1996) Cloning and functional expression of cDNAs encoding human and rat pancreatic polypeptide receptors. *Proc Natl Acad Sci USA* **93:**4661–4665.

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