

## Cloned human neuropeptide Y receptor couples to two different second messenger systems

HERBERT HERZOG, YVONNE J. HORT, HELEN J. BALL, GILLIAN HAYES, JOHN SHINE, AND LISA A. SELBIE

Garvan Institute of Medical Research, St. Vincent's Hospital, 384 Victoria Street, Darlinghurst, Sydney, NSW 2010, Australia

Communicated by W. J. Peacock, March 12, 1992 (received for review December 12, 1991)

**ABSTRACT** Neuropeptide Y (NPY) is one of the most abundant neuropeptides in the mammalian nervous system and exhibits a diverse range of important physiological activities, including effects on psychomotor activity, food intake, regulation of central endocrine secretion, and potent vasoactive effects on the cardiovascular system. Two major subtypes of NPY receptor (Y1 and Y2) have been defined by pharmacological criteria. We report here the molecular cloning of a cDNA sequence encoding a human NPY receptor and the corrected sequence for a rat homologue. Analysis of this sequence confirms that the receptor is a member of the G protein-coupled receptor superfamily. When expressed in Chinese hamster ovary (CHO) or human embryonic kidney (293) cells, the receptor exhibits the characteristic ligand specificity of a Y1 type of NPY receptor. In the 293 cell line, the receptor is coupled to a pertussis toxin-sensitive G protein that mediates the inhibition of cyclic AMP accumulation. In the CHO cell line, the receptor is coupled not to the inhibition of adenylate cyclase but rather to the elevation of intracellular calcium. These results demonstrate that second messenger coupling of the NPY-Y1 receptor is cell type specific, depending on the specific repertoire of G proteins and effector systems present in any cell type.

Neuropeptide Y (NPY), a 36-amino acid peptide, is an important regulator in both the central and peripheral nervous systems (1). NPY is highly conserved in primary structure between species, as the sequences of human, rat, rabbit, and guinea pig are identical and differ from the porcine sequence by only a single amino acid (2). NPY also shares close sequence homology and a common tertiary structure with a family of peptides which include peptide YY (PYY) and pancreatic polypeptide (PP) (3). Studies with peptide fragments of NPY have indicated that multiple NPY receptor subtypes exist (4). The two major receptor subtypes have been designated Y1 and Y2, with the Y1 receptor having the ability to respond to an analog of NPY modified at residues 31 and 34 ([Leu<sup>31</sup>,Pro<sup>34</sup>]NPY) (5). The Y2 receptor subtype is defined on the basis of its affinity for the NPY peptide carboxyl-terminal fragment NPY-(13–36) (4). NPY binding sites have been identified in a variety of tissues, including brain (6), spleen (7), intestinal membranes, and aortic smooth muscle cells (8). In addition, binding sites have been reported in a number of rat and human cell lines (9). Both the Y1 and Y2 receptors are members of the G protein-coupled receptor superfamily and act by transducing the signal caused by the binding of NPY to an intracellularly located protein complex that binds GTP. This G protein complex in turn activates a variety of second messenger systems, including a decrease in cyclic AMP and an increase in intracellular calcium (10). However, there are reports of NPY receptors coupled to phosphoinositol metabolism, suggesting the existence of additional receptor subtypes and/or multiple functions for the Y1 and Y2 subtypes (6, 11).

We report here the molecular cloning of a cDNA sequence encoding a human NPY receptor,\* which exhibits the characteristic ligand specificity of a Y1 receptor. When expressed in different cell lines, the receptor couples via pertussis toxin-sensitive G proteins to different second messenger systems.

### MATERIAL AND METHODS

**Nucleotide Sequence Determination.** Total RNA (3 µg) from rat brain was used as a template to synthesize random primed single-stranded cDNA. The cDNA was used in a polymerase chain reaction (PCR) together with the oligonucleotide primers, which correspond to positions 672–584 and 48–78 in the rat cDNA clone FC5 (12). PCR conditions: 30 cycles at 95°C for 1 min, 63°C for 2 min, and 72°C for 1 min. The reaction product was digested with *Eco*RI and *Pst* I, gel purified, and subcloned for sequencing in the Bluescript vector (Stratagene) for confirmation of the sequence.

λ cDNA libraries derived from human fetal brain (P. Seeburg, University of Heidelberg) and human adult hippocampus (Stratagene) (9 × 10<sup>5</sup> plaque-forming units each) were screened with <sup>32</sup>P-labeled rat cDNA as a probe under stringent hybridization conditions. Three strongly hybridizing clones were isolated and the cDNA inserts were subcloned for sequencing in Bluescript vectors. The largest cDNA [HY1, 2.5 kilobases (kb)] contains an open reading frame of 384 amino acids encoding the human NPY receptor subtype Y1. The two other clones (F5 and F13) are truncated versions of the same cDNA with 100% nucleotide identity in the overlapping regions (positions 664–1555 and 670–1925), respectively.

**Binding Assay.** The coding region of the NPY-Y1 receptor was subcloned in the pcDNA NEO expression vector (Invitrogen, San Diego) to generate pHZ30 and transfected into the Chinese hamster ovary (CHO) K1 (American Type Culture Collection CCL 61) and the human embryonic kidney 293 (American Type Culture Collection CRL 1573) cell lines by using a modified calcium phosphate transfection method (13). CHO cells were maintained under 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (1:1) and 293 cells were grown in minimal essential medium (MEM) with Hanks' salts, and both media contained 2 mM glutamine, 100 international units of penicillin, streptomycin at 100 µg/ml, and 10% fetal calf serum. Human neuroblastoma SK-N-MC cells (American Type Culture Collection HTB10) were cultured in DMEM/Ham's F-12 (1:1) with the above additions and including 0.1 mM MEM nonessential amino acids. All media and materials for tissue culture were from Cytosystems (Castle Hill, N.S.W., Australia). Stably transfected cells were selected with neomycin and tested for the ability to bind NPY/PYY peptide analogues. Transfected cells (1 × 10<sup>6</sup>) were incubated in 0.5 ml of assay buffer [50 mM

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Abbreviations: NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide.

\*The nucleotide sequence of the human neuropeptide Y-Y1 receptor cDNA clone HY1 has been deposited in the GenBank data base (accession no. M84755) and will be published elsewhere.

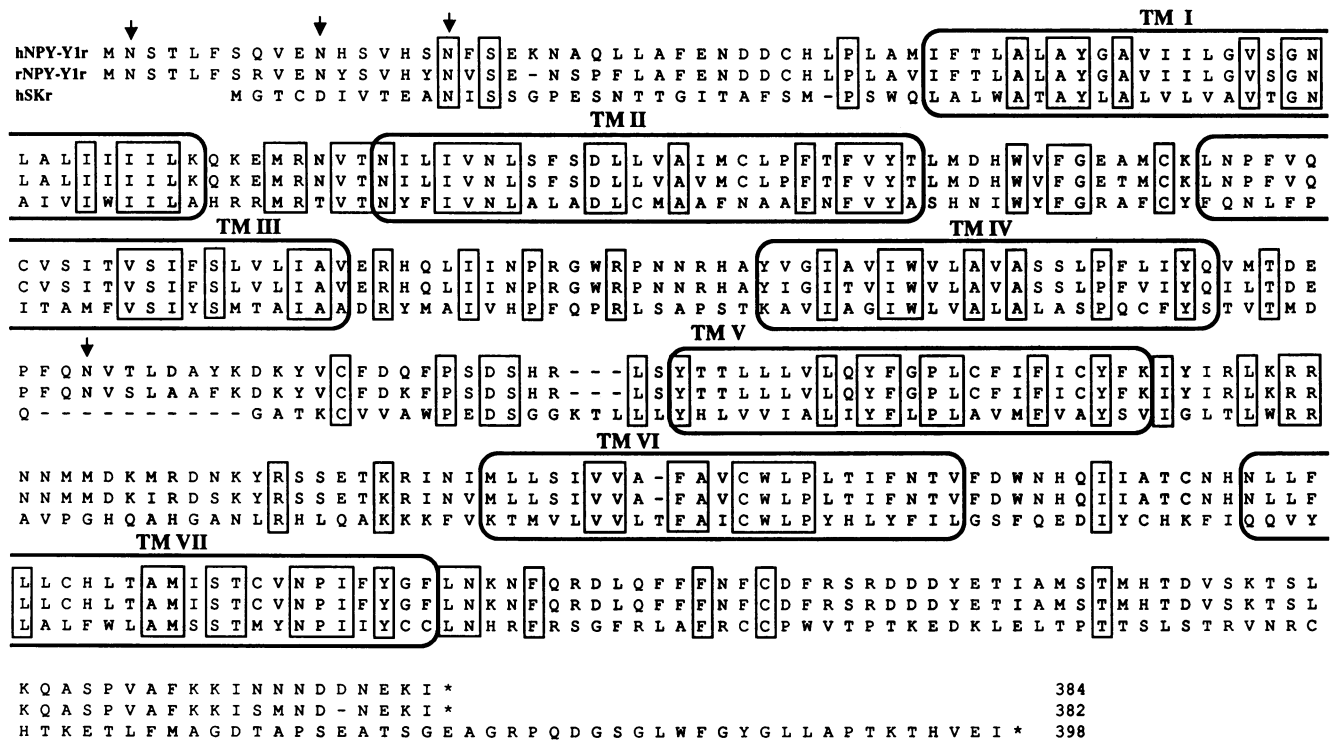


Fig. 1. Alignment of amino acid sequences for human NPY-Y1 receptor (hNPY-Y1r), rat NPY-Y1 receptor (rNPY-Y1r) (12), and human substance K receptor (hSKr) (16). The boxed amino acids represent residues that are identical in all three sequences. Dashes show deletions of the amino acid residues compared with the other sequences. Putative transmembrane segments (TM I-VII) of the receptor are indicated by bold frames, with the termini of each segment tentatively assigned on the basis of a hydrophobicity analysis. Arrows indicate potential N-glycosylation sites. Sequence identity within the first 350 amino acids between hNPY-Y1r and hSKr is 30%.

Tris·HCl, pH 7.4/2 mM CaCl<sub>2</sub>/5 mM KCl/120 mM NaCl/1 mM MgCl<sub>2</sub>/0.1% bovine serum albumin (Sigma) in the presence of 0.05 nM <sup>125</sup>I-labeled NPY (<sup>125</sup>I-NPY; Dupont/NEN) and increasing concentrations of human NPY or related peptides (Auspep, Melbourne, Victoria, Australia). Cells were incubated with the radiolabeled peptide and competitor peptides for 1 hr at room temperature and pelleted in a microcentrifuge for 4 min. Radioactivity was measured for 1 min in a  $\gamma$  counter.

**Calcium Measurements.** Cells transfected with the human NPY-Y1 receptor or SK-N-MC cells were suspended in loading medium (modified RPMI 1640 medium/10 mM Hepes/1% newborn calf serum) and incubated in a spinner flask at 37°C for 2.5 hr at 1 × 10<sup>6</sup> cells per ml. Cells were then treated with 1  $\mu$ M Fura-2 acetoxyethyl ester (fura-2 AM; Molecular Probes) for 30 min at 37°C, washed twice with loading medium, and resuspended at 5 × 10<sup>6</sup> cells per ml. Immediately before fluorescence spectroscopy, cells were recovered by centrifugation at 1000 rpm and resuspended at 1 × 10<sup>6</sup> cells per ml in a modified Krebs buffer (135 mM NaCl/4.7 mM KCl/1.2 mM MgSO<sub>4</sub>/1.2 mM KH<sub>2</sub>PO<sub>4</sub>/5 mM NaHCO<sub>3</sub>/1 mM CaCl<sub>2</sub>/2.8 mM glucose/10 mM Hepes, pH 7.4) containing 1 mM sulfinpyrazone. Bombesin was purchased from Sigma and Auspep. Fluorescence recordings were made on a Hitachi fluorescence spectrometer (F4010) at 340 nm (excitation) and 505 nm (emission) over 10 min with slit widths of 5 nm and a response time of 2 sec. Intracellular calcium was quantitated by using equations described by Grynkiewicz *et al.* (14).

**Cyclic AMP Assay.** Cyclic AMP was assayed in whole cells treated for 15 min at 37°C with 100  $\mu$ M isobutylmethylxanthine (IBMX; Sigma). Transfected 293 cells or CHO cells (1 × 10<sup>6</sup>/0.5 ml reaction) were incubated with 10  $\mu$ M forskolin and various concentrations of [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY or PYY-(13-36) for 15 min at 37°C. Reactions were terminated with the addition of HCl to 0.1 M, incubation at room temperature

for 15 min, and neutralization and sample dilution in 50 mM sodium acetate, pH 6.2. Cyclic AMP was quantitated by using a radioimmunoassay (Dupont/NEN).

**RESULTS AND DISCUSSION**

**Molecular Cloning of the Human NPY-Y1 Receptor cDNA.** Recently, a cDNA sequence encoding a putative member of

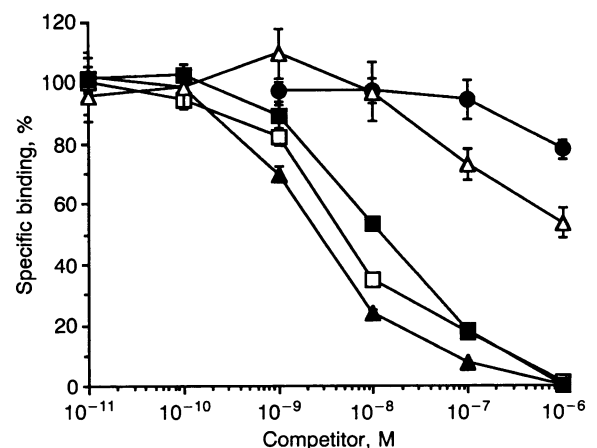


Fig. 2. Inhibition of porcine <sup>125</sup>I-PYY binding with various NPY-related peptides. Increasing concentrations of human NPY (□), human [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY (■), porcine PYY (▲), PYY-(13-36) (●), and human PP (△) were tested for their ability to inhibit the binding of <sup>125</sup>I-PYY. Results are expressed as a percentage of the maximal specifically bound radiolabeled PYY. Standard errors of triplicate samples are shown. Similar values for NPY binding were obtained with transfected 293 cells and with the human neuroblastoma SK-N-MC cell line. Untransfected CHO and 293 cells showed no specific binding of NPY.

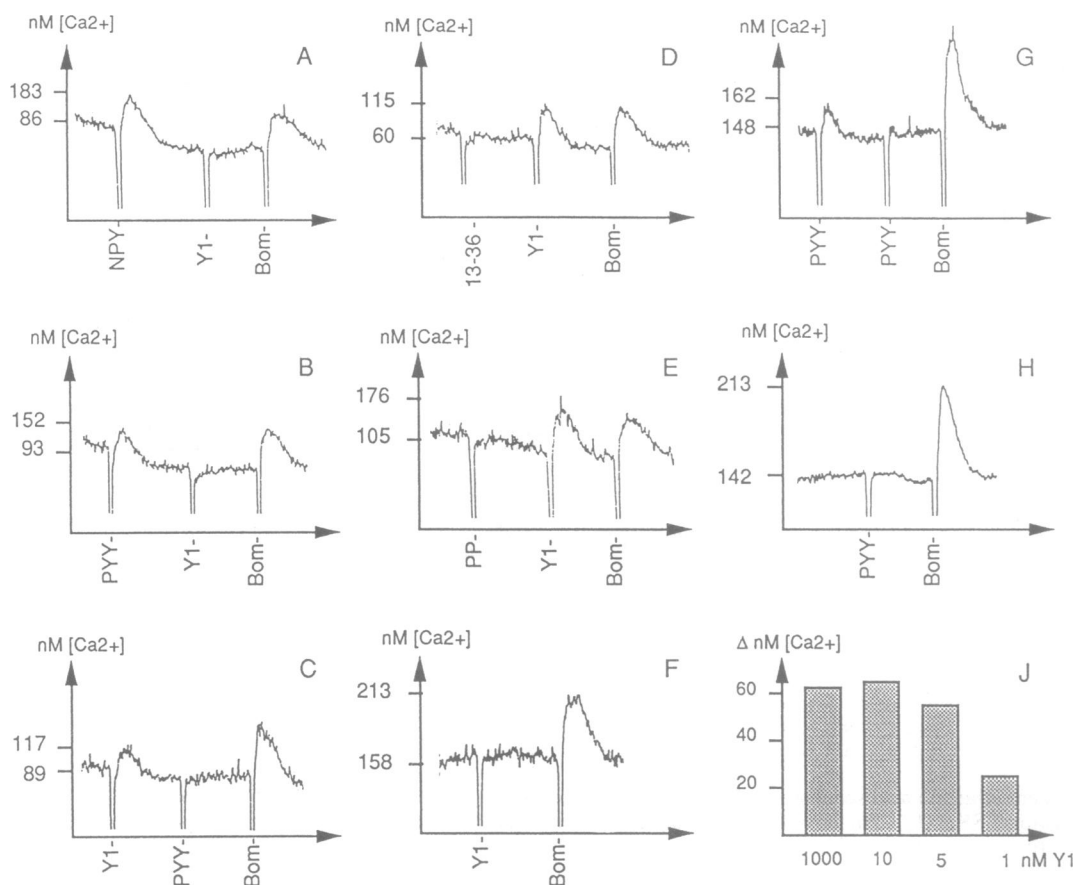


FIG. 3. Stimulation of intracellular calcium response mediated by the NPY-Y1 receptor. Intracellular calcium levels of fura-2-loaded CHO cells were measured in response to NPY, [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY (Y1), PYY, PYY-(13-36) (13-36), or PP. After successive addition of two of these compounds the intracellular calcium response mediated by the endogenous bombesin receptor was measured with the addition of 1  $\mu$ M bombesin (Bom) (Auspep). The NPY-Y1 receptor-expressing CHO cell line was stimulated with 100 nM NPY (A), PYY (B), [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY (C), PYY-(13-36) (D), or PP (E). Untransfected CHO cells did not respond to any of the NPY analogues, including 100 nM [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY (F). Treatment of stable transfected cells overnight with pertussis toxin at 100 ng/ml abolished the response to 2.5  $\mu$ M PYY (H), as compared with untreated cells (G), but did not affect the intracellular response to 1  $\mu$ M bombesin. The intracellular calcium increase mediated by the NPY-Y1 receptor was dependent on the concentration of [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY and was maximal at 10 nM (J).

the G-coupled receptor family was isolated from rat brain RNA (12). The distribution of this mRNA in the central nervous system is similar to that predicted for NPY binding sites (15). We used the PCR to amplify sequences of this cDNA from rat brain RNA as a probe to screen human hippocampal and fetal brain cDNA libraries. Several strongly hybridizing clones were isolated and sequenced, with the longest of these (HY1, 2.5 kb) encoding a complete open reading frame of 384 amino acids with high homology to the rat receptor sequence (Fig. 1). The sequence shown for the rat receptor is a corrected version of the published sequence for the cDNA clone FC5 (12). Sequence analysis of a PCR fragment (nucleotides 643-1191) encoding the carboxyl-terminal sequence of the rat receptor, obtained from the pheochromocytoma cell line PC12 (17), demonstrated three differences from the published sequence. These introduced two frame shifts by deleting G at position 1031 and 1034 and inserting G at position 1098. The corrected rat receptor sequence shows 94% identity to the human HY1 sequence. Analysis of the amino acid sequence of HY1 shows features typical of G protein-coupled receptors (10). These include seven hydrophobic putative membrane-spanning regions as well as potential glycosylation sites (Fig. 1). Several serine and threonine residues, candidates for phosphorylation, are found in the carboxyl-terminal sequence. Amino acid residues highly conserved among different G protein-coupled receptors are also present (e.g., Arg<sup>138</sup>) (Fig. 1). Comparison of the amino acid sequence with the other G protein-coupled

receptor sequences identifies the human substance K receptor (16) as the most closely related sequence, with 30% identity (Fig. 1).

**Analyses of Ligand Specificity.** To examine the ligand binding properties and second messenger coupling of the receptor encoded by HY1, the coding region was subcloned in a mammalian cell expression vector and transfected into CHO and 293 cell lines. Stable transformed cell lines were selected for detailed analysis. Both types of cell line expressing the HY1 clone exhibit specific binding of NPY. Scatchard analysis of NPY binding indicates the presence of high- and low-affinity sites for NPY, typical for coupling of the receptor to a G protein or proteins in these cells. The calculated  $K_D$  values for these sites are approximately 5.1 nM and 198 nM, respectively (data not shown). These values are similar to the binding affinities reported for specific NPY binding to various peripheral tissues and cell lines (18, 19), consistent with its classification as a NPY receptor. NPY receptor subtypes have been pharmacologically defined by their affinity for a range of related peptide analogues. NPY and PYY, members of the PP family, have nearly identical affinities for the cloned receptor (Fig. 2), whereas PP has virtually no affinity at physiological concentrations. A NPY analogue, [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY, with known selectivity for Y1 receptors (5), has a high affinity for the expressed receptor ( $IC_{50}$  = 10 nM), while the Y2 selective analogue PYY-(13-36) has almost no affinity (Fig. 2). This ligand specificity and rank order of potency—NPY = PYY > [Leu<sup>31</sup>,Pro<sup>34</sup>]NPY >> PP > PYY-

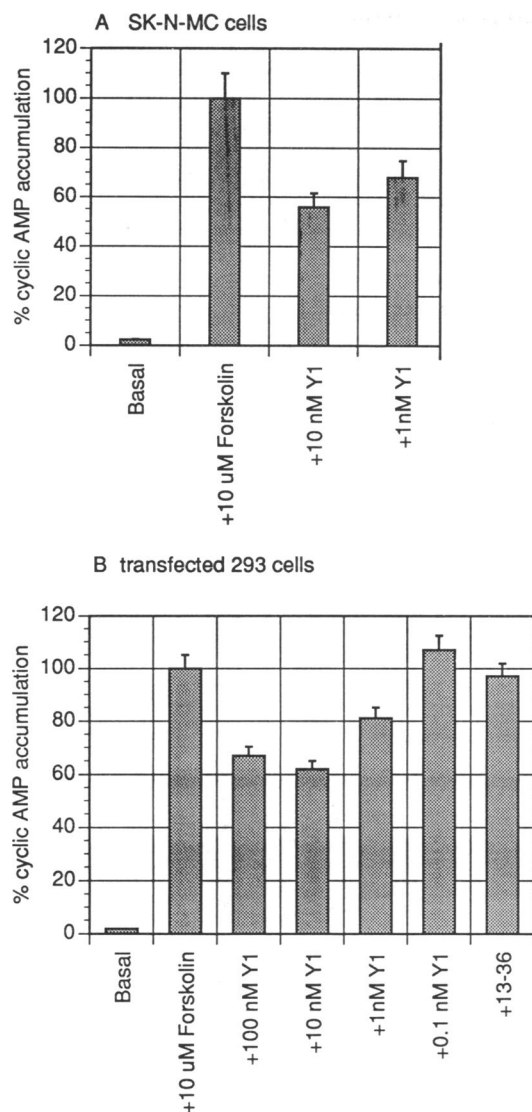


FIG. 4. [ $\text{Leu}^{31}$ , $\text{Pro}^{34}$ ]NPY inhibition of cyclic AMP accumulation in SK-N-MC (A) cells and 293 cells transfected with the human NPY-Y1 receptor cDNA (B). The cyclic AMP response to [ $\text{Leu}^{31}$ , $\text{Pro}^{34}$ ]NPY (Y1) or porcine PYY-(13-36) (13-36) is expressed as a percentage of the cyclic AMP level produced with 10  $\mu$ M forskolin from three independent experiments. Basal levels were  $2.5 \pm 0.13$  pmol per  $10^6$  cells (untransfected 293 cells),  $2.26 \pm 0.6$  pmol per  $10^6$  cells (transfected 293 cells), and  $1.8 \pm 0.55$  pmol per  $10^6$  cells (SK-N-MC cells). Cyclic AMP levels in cells stimulated by 10  $\mu$ M forskolin were  $121.2 \pm 19.8$  pmol per  $10^6$  cells (untransfected 293 cells),  $139 \pm 14.2$  pmol per  $10^6$  cells (transfected 293 cells), and  $137.6 \pm 1.85$  pmol per  $10^6$  cells (SK-N-MC cells).

(13-36)—clearly demonstrate that the HY1 clone encodes a Y1 subtype of the NPY/PYY receptor family.

**Increases in Intracellular Calcium in Stable Transfected CHO Cells.** To analyze the functional coupling of the cloned receptor in CHO K1 and 293 cells, we measured the effects of NPY on intracellular calcium levels and cyclic AMP accumulation. In the CHO cell line, stimulation with either NPY or PYY results in an immediate and substantial increase in intracellular calcium (Fig. 3). This effect can be abolished by preincubation with pertussis toxin, suggesting receptor interaction with a pertussis toxin-sensitive G protein. The calcium response is receptor specific, being elicited by NPY, PYY, and the Y1 agonist but not by PP or PYY-(13-36). The NPY-induced rise in intracellular calcium is comparable to that observed in the human neuronal cell line SK-N-MC,

which contains only the Y1 specific NPY receptor subtype (18) (data not shown), as well as in vascular smooth muscle cells (19). However, in the 293 cell line no increase in intracellular calcium was observed after stimulation by NPY, PYY, or [ $\text{Leu}^{31}$ , $\text{Pro}^{34}$ ]NPY.

**Inhibition of Adenylate Cyclase Activity in Stably Transfected 293 Cells.** The majority of NPY receptors in the brain and peripheral tissue couple to inhibition of adenylate cyclase and hence decrease cyclic AMP (20). As shown in Fig. 4A, NPY decreases forskolin-stimulated cyclic AMP levels in SK-N-MC cells. Interestingly, NPY or PYY had no specific effect on the inhibition of cyclic AMP accumulation (either basal or stimulated by forskolin) in the HY1-expressing CHO cell line. This suggests that either this subtype is not normally coupled to this second messenger system or, more likely, CHO cells lack the appropriate  $G_{i/o}$  isoform. The latter explanation is supported by the demonstration that, in contrast to CHO cells, transfected 293 cells exhibit a clear inhibition of cyclic AMP accumulation (Fig. 4B). The inhibition of the cyclic AMP accumulation was Y1 specific and the effect was maximal at 10 nM NPY. Preincubation with pertussis toxin abolished the effect.

**Conclusions.** On the basis of both receptor pharmacology and the effects on intracellular calcium and cyclic AMP accumulation, the sequence encoded by HY1 represents a Y1 subtype of a human NPY/PYY receptor. The demonstration that this receptor couples to different second messenger systems in a cell type specific manner suggests that the functional diversity exhibited by NPY may result not only from the presence of different receptor subtypes but also from the restricted availability of second messenger systems in different cell types. Knowledge of the primary structure and the second messenger coupling of the NPY-Y1 receptor subtype is particularly important given the role of this subtype in control of cardiovascular parameters (21). In this context, these results should facilitate the development of therapeutic drugs important in blood-pressure control and other cardiovascular diseases. Using this sequence for isolation of other related receptor subtypes and evaluation of signal transduction systems in other cell lines should shed considerable light on the molecular mechanisms involved in the NPY-mediated control of a variety of physiological responses.

We gratefully acknowledge the expert advice of A. P. Czernilofsky and T. Biden and the assistance of C. Browne and M. Lui. This work was supported by the National Health and Medical Research Council and Boehringer Ingelheim (Bender & Co., Austria). H.H. is a fellow of the E. Schrödinger Foundation (Austria).

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