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ACCELERATED COMMUNICATION

Neuropeptide Y_1 Subtype Pharmacology of a Recombinantly Expressed Neuropeptide Receptor

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SUMMARY

Neuropeptide Y (NPY) is an important central and peripheral modulator of neural and endocrine functions. This neuropeptide interacts with at least two pharmacologically distinct receptors, termed Y_1 and Y_2. At Y_1 receptors, the NPY analog [Leu^{31},Pro^{34}]NPY, but not the carboxyl-terminal fragment NPY-(18–36), displaces radiolabeled NPY and the sequence-related peptide YY, whereas Y_2 receptors exhibit the opposite selectivity. We have used cultured mammalian 293 cells for the high level transient expression of a previously cloned putative neuropeptide receptor of rat brain. We report that this receptor displays the ligand binding properties and selectivity of a Y_1 receptor, with a single high affinity site for ^{125}I-NPY (K_d, 0.7 ± 0.2 nM). The functionality of the recombinantly expressed receptor was demonstrated by an inhibition of adenylyl cyclase and a concomitant mobilization of intracellular Ca^{2+}.

NPY, a peptide of 36 amino acids, was first isolated from porcine brain (1). It belongs to a family of structurally related, centrally and peripherally expressed peptides that includes PYY and PP. NPY is one of the most abundant neuropeptides in the mammalian brain, where it participates in the control of psychomotor activities, cognitive functions, circadian rhythm, food intake, and neuroendocrine regulation (2, 3).

NPY is thought to target two distinct receptors, termed Y_1 and Y_2 (4). The analogs [Leu^{31},Pro^{34}]NPY and [Pro^{34}]NPY are specific agonists at Y_1 receptors, whereas the two carboxyl-terminal fragments NPY-(13–36) and NPY-(18–36) act preferentially on Y_2 receptors (4–7). Y_1 receptors are found in brain (8, 9), aortic membranes (9), and rat PC-12 pheochromocytoma and human SK-N-MC neuroblastoma cell lines (7, 10, 11). Y_2 receptors exist in rabbit kidney and pig spleen membranes, rat vas deferens (8, 12, 13), and the human SMS-MSN neuroblastoma cell line (10). The existence of an additional NPY receptor subtype, Y_3, has been postulated on the basis of higher affinity for NPY than PYY (14), but an unequivocal identification of this receptor subtype awaits the availability of a selective ligand. The affinity of NPY for its receptors is GTP dependent, and NPY binding results in either inhibition or stimulation of adenylyl cyclase, depending on the tissue investigated (15–17).

Although NPY receptor subtypes have been characterized by using bioassays and ligand binding assays and were partially purified (12, 18–21), none have been expressed in recombinant form. The molecular cloning of a novel guanine nucleotide-binding protein-coupled receptor has recently been reported; its primary structure suggested that it may belong to the superfamily of neuropeptide receptors (22). Expression patterns of the receptor-encoding mRNA in rat brain revealed significant similarities to the localization of the Y_1 subtype of NPY receptors in autoradiographic ligand-binding studies (23, 24). We analyzed the ligand selectivity of this receptor and present evidence that it, indeed, constitutes a functional Y_1 subtype of NPY receptors.

Experimental Procedures

Materials. NPY, [Leu^{31},Pro^{34}]NPY, NPY-(18–36), phenylmethylsulfonyl fluoride, bacitracin, EDTA, and Triton X-100 were obtained from Serva (Heidelberg, Germany). Human PYY was from Bissendorf Biochemicals (Hannover, Germany), and human PP was purchased from Novabiochem. ^{125}I-labeled peptides were from NEN/DuPont. BSA, digitonin, carbachol, and 3-isobutyl-1-methylxanthine were from Sigma. Cell culture media, phosphate-buffered saline, and penicillin/

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ABBREVIATIONS: NPY, neuropeptide Y; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; K_d, dissociation constant; MEM, minimum essential medium; PYY, peptide YY; PP, pancreatic polypeptide; BSA, bovine serum albumin; hCG, human chorionic gonadotropin; IC_{50}, 50% inhibitory concentration; FCS, fetal calf serum.

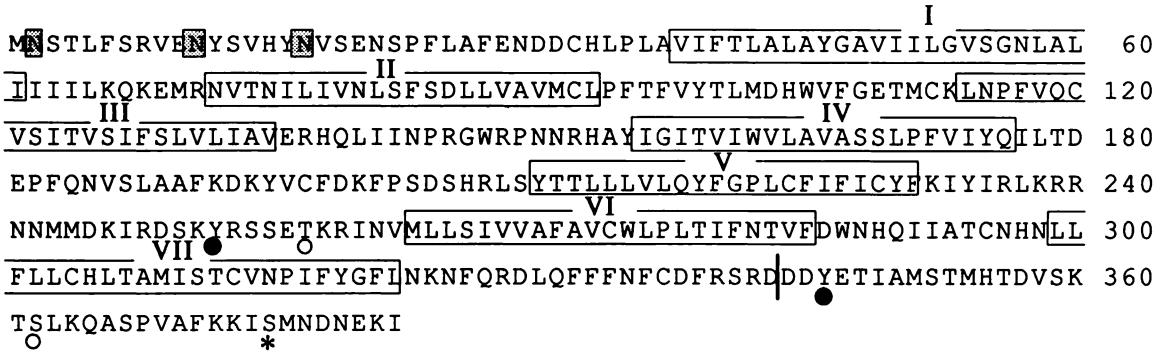

Fig. 1. Primary structure of a rat Y_1 receptor. The transmembrane segments I–VII are *boxed*, three potential *N*-linked glycosylation sites are *shaded*, and putative phosphorylation sites are denoted by *closed circles* (tyrosine kinase), *open circles* (kinase C), or an *asterisk* (kinase A). The *vertical bar* between residues 343 and 344 marks the divergence from the previously published carboxyl-terminal sequence (22).

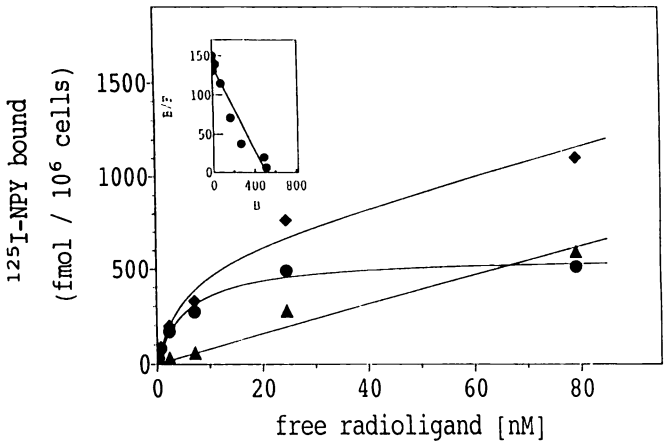

Fig. 2. Binding isotherms for ^{125}I-NPY with 293 cells transiently expressing a rat Y_1 receptor. Total (♦), nonspecific (▲), and specific (●) binding is shown for different concentrations of radioligand. *Inset*, Scatchard plot of the specific binding.

streptomycin solutions were purchased from GIBCO, and FCS was from Biospa. Fura-2 acetoxymethyl ester was from Calbiochem. Highly purified hCG (lot CR-123; 12,780 IU/mg) was a gift from the National Hormone and Pituitary Program of the National Institute of Diabetes and Diseases of the Kidney (Bethesda, MD).

Plasmid constructs. For expression studies, the entire coding region of the FC5 receptor was inserted, as a *Bgl*II-*Xba*I fragment (nucleotides −67 to 1328) (22), into a cytomegalovirus promoter-based expression vector (25). Sequencing of the expression plasmid pRFC5 revealed three nucleotide errors, compared with the previously published FC5 cDNA sequence, the correction of which led to a frame-shifted carboxyl terminus of 32 amino acid residues for the encoded receptor protein. The corrected cDNA sequence is available in the EMBL/GenBank database, under the accession number Z 11504, and the deduced amino acid sequence of the receptor is displayed in Fig. 1.

Peptide binding. Human embryonic kidney 293 cells (ATCC CRL 1573) were seeded to a density of 5×10^4 cells/well in 24-well plates, cultured in Dulbecco's modified Eagle medium/nutrient mixture F-12 (Ham) (1:1) containing 10% FCS and 100 units/ml antibiotics, and transfected (26) using 0.5 μg of pRFC5 plasmid DNA/well. For binding assays, medium was removed 24 hr after transfection, and the cells were washed once with 0.5 ml of MEM/25 mM HEPES. Incubation medium (300 μl of MEM/25 mM HEPES containing 0.5% BSA, 50 μM phenylmethylsulfonyl fluoride, and 0.1% bacitracin) was then added and the samples were incubated for 30 min. The addition of radiolabeled peptide (12 pM) and increasing amounts of either unlabeled NPY, [Leu^{31},Pro^{34}]NPY, NPY-(18–36), PYY, or PP was followed by a 3-hr incubation period, with gentle shaking. The supernatants were removed and the cells were washed twice with phosphate-buffered saline containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+}. The cells were then lysed in the same buffer containing 1% Triton X-100, lysates were resuspended, the wells were washed once with 0.25 ml of lysis buffer, and the radioactivity in the combined fractions was determined by liquid scintillation. All binding assays were performed in triplicate at room temperature.

cAMP accumulation. To determine cAMP levels, 293 cells were grown in MEM with Earle's salts, supplemented with antibiotics and 10% heat-inactivated FCS. Cells exponentially growing in 10-mm culture wells were transfected at 50–70% confluency, as described earlier (26), using a total of 0.5 μg of recombinant expression vectors containing the rat lutropin receptor cDNA (pCLHR) (27) and the FC5 receptor cDNA (pRFC5). The growth medium was replaced by fresh medium 18 hr after transfection, and 16 hr later the cells were preincubated for 10 min in 0.5 ml of growth medium containing 0.1 mM 3-isobutyl-1-methylxanthine, to inhibit phosphodiesterase. Receptor stimulation was performed for 20 min at 37° in the presence of hCG (0.5 nM) and [Leu^{31},Pro^{34}]NPY, as well as in the absence of the neuropeptide. Stimulation was terminated by freezing and thawing of the cells. Protein was precipitated by the addition of 1 ml of absolute ethanol, and cell debris was removed by centrifugation. Aliquots (5–20 μl) from the supernatants were lyophilized and assayed for cAMP by using the Amersham RPA 508 assay system. cAMP levels were calculated using a calibration curve determined in parallel by the addition of cyclic nucleotide to control samples in the range between 0 and 1600 fmol.

Ca^{2+} measurements. 293 cells were seeded at a density of 1×10^5 cells/well in six-well plates and were transfected with plasmid pRFC5 as described above. The medium was removed 3–5 days after transfection, and the cells were washed once with 1 ml of MEM/25 mM HEPES, pH 7.4. The cells in each well were then overlayed with 2 ml of MEM/25 mM HEPES, pH 7.4, containing 0.5% BSA, and fura-2 acetoxymethyl ester was added to a final concentration of 100 nM. After incubation in the dark for 1 hr at room temperature, the cells were washed with a buffer containing 10 mM HEPES, pH 7.4, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, and 150 mg/liter BSA and were overlayed with 2 mM HEPES buffer. The specific fluorescence of individual cells was determined in the Zeiss microscope-photometer system (MPM type fast fluorescence photometry, with excitation at 340/380 nm and emission at 510 nm), before and after the cells were exposed to NPY (1 μM) and carbachol (50 μM).

Results and Discussion

An expression vector carrying the cloned FC5 receptor cDNA (pRFC5) was transiently expressed in human embryonic kidney 293 cells. The cDNA encodes a polypeptide sequence of 34.4 kD (Fig. 1), with three potential *N*-glycosylation sites in the

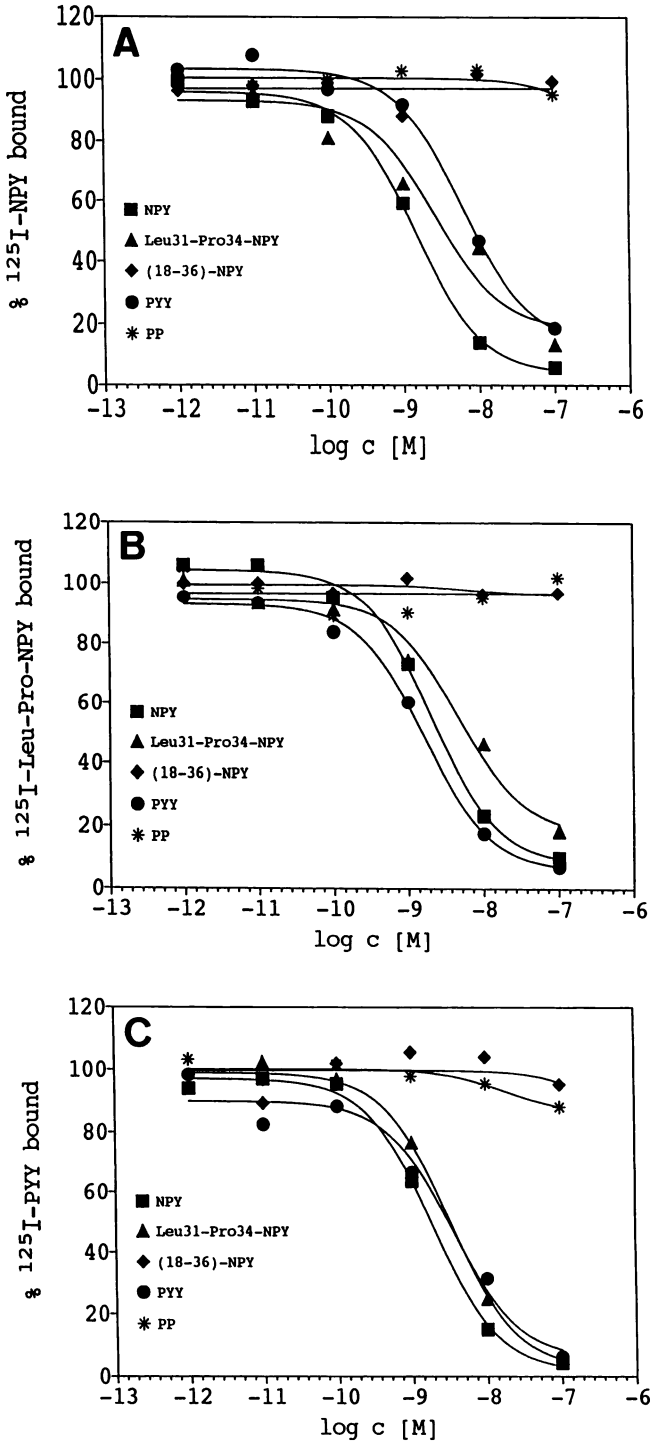

Fig. 3. Ligand specificity of a recombinantly expressed Y_1 receptor. For each of three radioligands, ^{125}I-NPY (A), ^{125}I-[Leu^{31},Pro^{34}]NPY (B), and ^{125}I-PYY (C), displacement curves were established using NPY, PYY, [Leu^{31},Pro^{34}]NPY, NPY-(18–36), and PP as the competing ligands. The values for triplicate experiments performed with the three radioligands with each peptide are listed in Table 1. Standard deviations were in the range of 14 ± 4% for specific and 1 ± 0.5% for nonspecific binding.

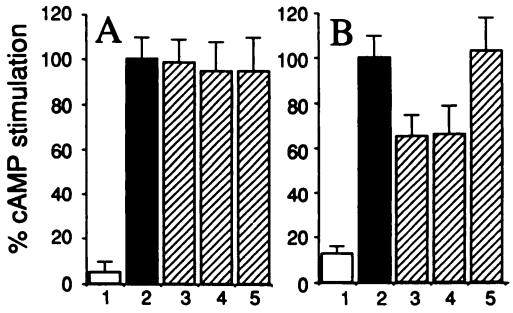

Fig. 4. Inhibition of luteinizing hormone-stimulated cAMP accumulation. Adherent 293 cells transiently expressing lutropin/hCG receptor (A) or coexpressing lutropin/hCG receptors and Y_1 receptors (B) were treated with 0.5 nM hCG (*bars 2–5*), in the absence (*bar 2*) or presence (*bars 3–5*) of various concentrations of the Y_1 subtype-specific analog [Leu^{31},Pro^{34}]NPY. The concentrations used were 25 nM (*bar 3*), 0.25 nM (*bar 4*), and 2.5 pM (*bar 5*). cAMP was measured 20 min after receptor stimulation, as described in Experimental Procedures. *Bar 1*, cAMP levels in untreated cells. Maximal cAMP levels obtained in the absence of NPY were arbitrarily set to 100%. In three independent experiments, these levels corresponded to 300 ± 40 fmol/10^3 cells. Accordingly, the error bars comprise 20% of the determined cAMP values.

amino-terminal extracellular domain. Other consensus sequences for posttranslational modifications include four target sites for protein kinases within the third intracellular loop and the intracellularly located carboxyl terminus. Two potential phosphorylation sites for protein kinase C are located at residues threonine-257 and serine-362, two sites for tyrosine kinase at tyrosine-252 and -346, and one site for protein kinase A at serine-375. The presence of these sites suggests that the receptor may be regulated by phosphorylation (28).

Cells expressing FC5 receptor cDNA were analyzed for NPY receptor pharmacology. Whereas untransfected 293 cells did not bind ^{125}I-NPY, transfected cells exhibited specific, steady state binding of this radioligand within 3 hr. Saturation binding studies revealed a single, high affinity, binding site, with a K_d of 0.7 ± 0.2 nM and average B_{max} values of 2.7 ± 1.5 × 10^5 binding sites/cell (Fig. 2).

To examine ligand selectivity, we investigated the ability of the Y_1 receptor-specific NPY analog [Leu^{31},Pro^{34}]NPY and the Y_2 receptor-specific carboxyl-terminal fragment NPY-(18–36), as well as human PYY and human PP, to compete for the binding of ^{125}I-NPY, ^{125}I-[Leu^{31},Pro^{34}]NPY, and ^{125}I-PYY. The inhibition curves for increasing concentrations of the competitors are shown in Fig. 3, and the corresponding apparent IC_{50} values for the competitors are summarized in Table 1. NPY, PYY, and [Leu^{31},Pro^{34}]NPY were approximately equipotent in competing for the binding of all radioligands. In contrast, the Y_2 receptor-specific ligand NPY-(18–36) and human PP exhibited negligible affinities for the FC5 receptor. The rank order of relative potencies for the displacement of the radioligands ^{125}I-NPY and ^{125}I-PYY by NPY, the analog [Leu^{31},Pro^{34}]NPY, PYY, NPY-(18–36), and PP all correlated well with values previously reported for putative Y_1 binding sites (9, 12, 29). Thus, the high affinity for the selective analog [Leu^{31},Pro^{34}]NPY identifies the FC5 receptor as a Y_1 subtype of NPY receptors.

Activated NPY receptors of different tissues and cell lines have been described to inhibit adenylyl cyclase and to mobilize intracellular Ca^{2+} (15–17, 29–32). As shown in Fig. 4B, 293 cells transiently coexpressing the FC5 receptor and the G_s-coupled lutropin/hCG receptor (27) exhibited a 200-fold increase in intracellular cAMP after treatment with hCG. The hCG-mediated cAMP stimulation was markedly reduced when cells were simultaneously exposed to NPY. Hormone-induced cAMP accumulation in 293 cells not expressing the FC5 receptor was not affected by NPY treatment (Fig. 4A).

TABLE 1

Ligand affinities at the recombinantly expressed rat Y_1 receptor

Shown are the IC_{50} values (mean ± standard deviation) for the inhibition of ^{125}I-NPY, ^{125}I-[Leu^{31},Pro^{34}]NPY, and ^{125}I-PYY binding by NPY, PYY, PP, the NPY analog [Leu^{31},Pro^{34}]NPY, and the carboxyl-terminal fragment NPY-(18–36).

	IC_{50}		
	^{125}I-NPY	^{125}I-PYY	^{125}I-[Leu^{31},Pro^{34}]NPY
		M	
NPY	$1.59 \pm 0.65 \times 10^{-9}$ (9)[a]	$1.97 \pm 1.16 \times 10^{-9}$ (4)	$2.61 \pm 1.34 \times 10^{-9}$ (4)
PYY	$7.10 \pm 1.90 \times 10^{-9}$ (3)	$3.30 \pm 2.4 \times 10^{-9}$ (4)	$2.88 \pm 0.78 \times 10^{-9}$ (3)
[Leu^{31},Pro^{34}]NPY	$2.36 \pm 1.95 \times 10^{-9}$ (6)	$3.65 \pm 2.2 \times 10^{-9}$ (3)	$9.07 \pm 9.35 \times 10^{-9}$ (4)
PP	$>10^{-7}$ (3)	$>10^{-7}$ (3)	$>10^{-7}$ (3)
NPY-(18–36)	$>10^{-7}$ (5)	$>10^{-7}$ (3)	$>10^{-7}$ (3)

[a] Numbers in parentheses, numbers of experiments.

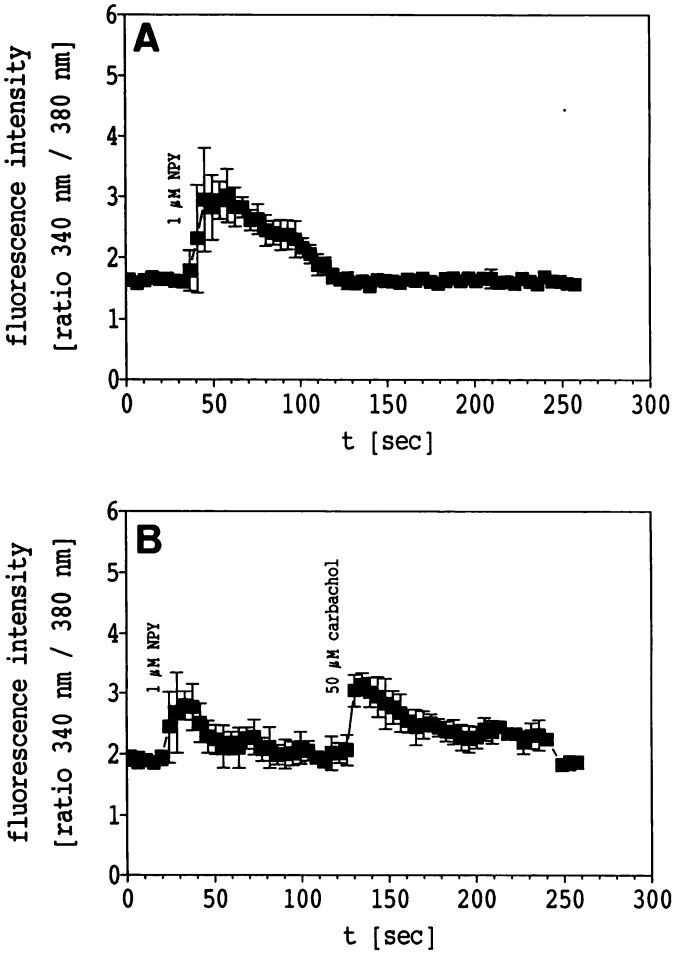

Fig. 5. Effect of NPY and carbachol on intracellular Ca^{2+} levels of 293 cells transiently expressing the rat Y_1 receptor. A, Response to NPY; B, signals obtained after consecutive stimulation of the same cells with NPY and carbachol. Fluorescence intensity is plotted against time. The traces show mean values ± standard deviations obtained from five individual cells.

To reveal a possible effect of NPY on intracellular Ca^{2+} levels, 293 cells transiently expressing the FC5 receptor were loaded with fura-2 and analyzed at the single-cell level for NPY-mediated Ca^{2+}-specific fluorescence changes. Fig. 5A documents that NPY leads to a transient rise in intracellular Ca^{2+}. Furthermore, consecutive stimulation by NPY and carbachol, a muscarinic agonist, generated independent Ca^{2+} signals of similar magnitude (Fig. 5B), indicating efficient signal transduction at both the recombinantly expressed Y_1 receptor and the endogenously expressed muscarinic receptor. The measurements shown were taken at various time points from single cells obtained from different transfection experiments. These cells expressed varying receptor numbers, resulting in fluctuations in the amounts of Ca^{2+} mobilized upon NPY stimulation. Nevertheless, unequivocal Ca^{2+} signals were recorded for each cell.

In summary, our data show that the heptahelical neuropeptide receptor FC5 exhibits the ligand binding specificity of a Y_1 subtype of NPY receptors. The functionality of this receptor was indicated by agonist-induced responses of expressing cells. Inhibition of adenylyl cyclase and Ca^{2+} mobilization have been reported for NPY-stimulated biological membranes (15–17, 24–32) and might reflect receptor coupling to a G_i protein. However, the responses in our *in vitro* system might be due, at least in part, to nonphysiological effects of high receptor expression (33). Detailed studies regarding signal transduction of the Y_1 receptor await the construction of stable cell lines with defined receptor numbers. The availability of this receptor should prove useful for dissecting its specific role in the NPY-mediated control of central and peripheral physiological functions.

Acknowledgments

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