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Biochimica et Biophysica Acta 1284 (1996) 134–137

BBA Biochimica et Biophysica Acta

Short sequence-paper

Cloning and functional expression of a cDNA encoding a mouse type 2 neuropeptide Y receptor [1]

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Received 8 July 1996; accepted 12 August 1996

Abstract

A cDNA clone homologous with the human neuropeptide Y (NPY)-Y2 receptor has been isolated from a mouse brain cDNA library. Analysis of the predicted amino-acid sequence indicates that the polypeptide encoded by this cDNA is 94% homologous to the human NPY-Y2 receptor. In Chinese hamster ovary (CHO) cells expressing the mouse NPY-Y2 receptor, an increase in intracellular Ca^{2+} and inhibition of forskolin-induced cAMP accumulation were observed due to stimulation with NPY, NPY-(13–36) and peptide YY, but not with pancreatic polypeptide or [Leu^{31},Pro^{34}]NPY. The fact that the NPY-induced increase in intracellular Ca^{2+} and inhibition of forskolin-induced cAMP accumulation were eliminated by pretreatment with pertussis toxin suggests that the NPY-Y2 receptor couples to PTX-sensitive G-protein(s), probably Gi/Go, in CHO cells.

Keywords: Molecular cloning; Neuropeptide Y; Y2-type receptor; (Mouse)

Neuropeptide Y (NPY) is an important regulator in the central and peripheral nervous system. The pancreatic polypeptide family includes NPY, pancreatic polypeptide (PP), and peptide YY (PYY), all of which are 36-amino-acid peptides characterized by a hairpin loop. NPY is widely distributed in the brain [1,2] and the peripheral nervous system [3], and is often co-localized with norepinephrine. NPY modulates numerous physiological processes, including appetite, anxiety, blood pressure, and circadian rhythm [4,5]. PYY is localized primarily in intestinal endocrine cells and functions as a circulating hormone with elevated postprandial levels; small amounts are also found in central and peripheral neurons [4,5]. Studies of various organs and cell types with peptide fragments of NPY have indicated that multiple NPY receptor subtypes exist. Based on a ranking of the pancreatic polypeptide family in order of potency to displace ^{125}I-NPY or ^{125}I-PYY binding, NPY receptors have been classified into at least four receptor subtypes [4,5]: (1) the Y1 type binds NPY, PYY, and an analog of NPY modified at residues 31 and 34 ([Leu^{31},Pro^{34}]NPY) > PP and the NPY peptide C-terminal fragment (NPY-(13–36)); (2) the Y2 type binds NPY, PYY, and NPY-(13–36) > PP and [Leu^{31},Pro^{34}]NPY; (3) the Y3 type binds NPY > PYY; and (4) the Y4 type binds PP > [Leu^{31},Pro^{34}]NPY > NPY.

Recently, Y1-type receptor cDNAs (human [6,7], mouse [8,9], rat [10], and *Xenopus laevis* [11]) and Y4-type receptor cDNAs (human [12,13] and mouse [14]) were isolated and found to have sequences similar to members of a G-protein coupled receptor superfamily. Moreover, Rose et al. [15] and Gerald et al. [16] cloned a human NPY receptor, which was similar in its ligand binding properties to the pharmacologically defined Y2 type. However, studies of tissue distribution using the human clone have been hindered by the limited availability of human tissues. Here we describe the homologous screening of a mouse Y2-type receptor. The clone has been functionally expressed in Chinese hamster ovary (CHO)-K1 cells where its activation mobilizes intracellular Ca^{2+} and inhibits forskolin-stimulated cAMP accumulation.

To obtain a mouse homologue of the NPY-Y2 receptor, a mouse brain cDNA library (λZAPII, $3 \cdot 10^5$ plaques)

Abbreviations: NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide; RT-PCR, reverse transcriptase-polymerase chain reaction; fura-2/AM, fura-2 pentaacetoxymethyl ester; CHO cells, Chinese hamster ovary cells; PTX, pertussis toxin; G-protein, guanine nucleotide-binding protein; kbp, kilobase pair(s); bp, base pair(s).

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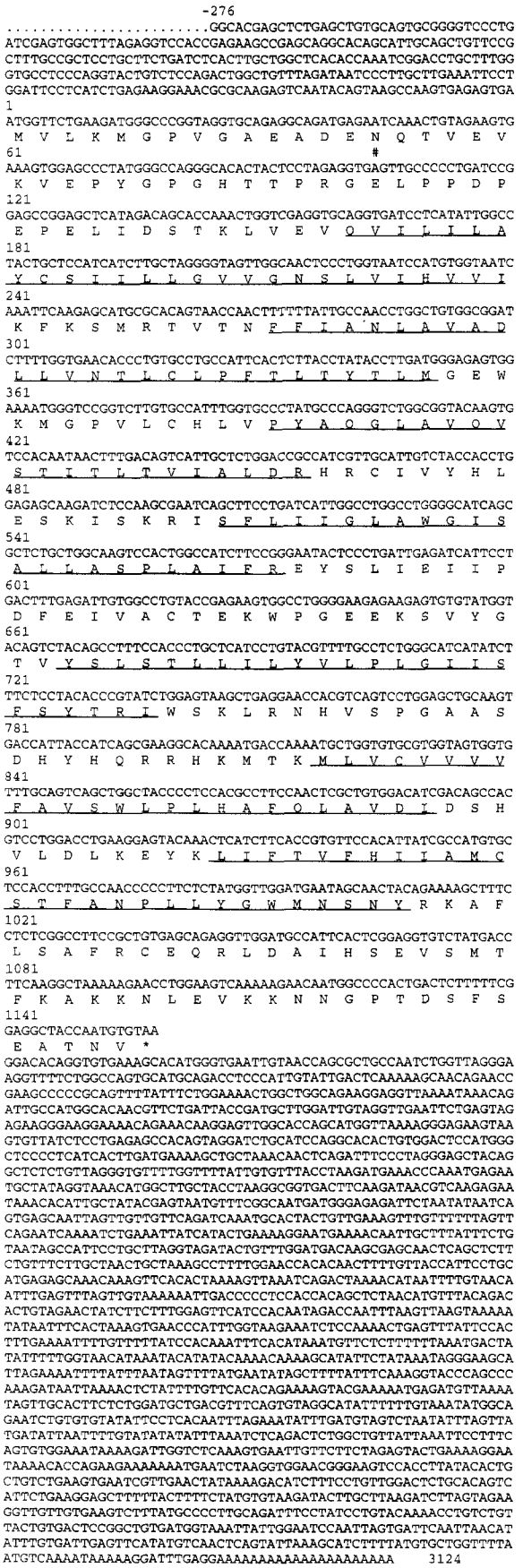
[1] The nucleotide sequence data reported in this paper have been submitted to the DDBJ/GenBank/EMBL Data Bank under the accession number D86238.

PII S0005-2736(96)00166-6



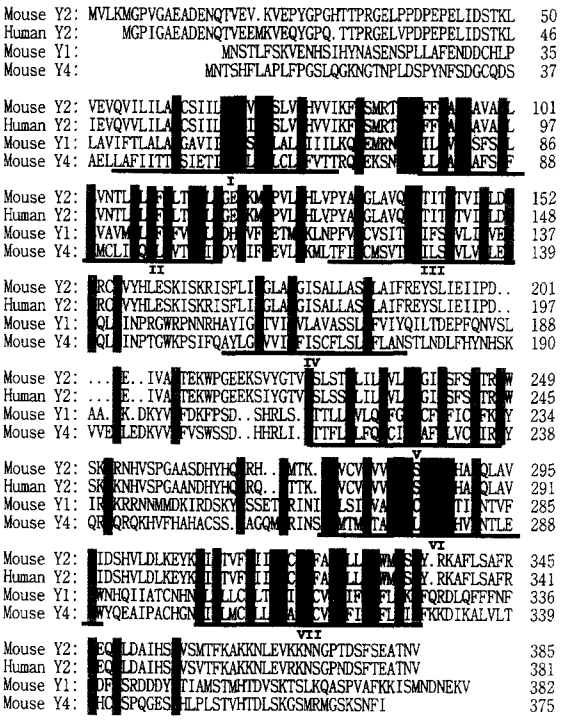


Fig. 2. Alignment of the predicted amino-acid sequence of the mouse Y2 receptor with the human Y2 [15,16], the mouse Y1 [8,9] and the mouse Y4 [4] receptor sequences. Periods represent spaces added for proper alignment. Numbers correspond to the amino-acid positions of each receptor. The seven putative transmembrane domains are underlined and numbered I–VII. Residues identical among the four receptors are shaded in gray.

was screened with a 867-base pair (bp) PCR-derived probe spanning amino acids 93–381 of the human NPY-Y2 receptor [6,7]. One positive clone, referred to in this report as mY2, has a 3400-bp insert DNA containing a 1155-bp open reading frame (Fig. 1). Since analysis of the predicted amino-acid sequence indicates that the polypeptide encoded by this cDNA has seven transmembrane regions typical of G-protein coupled receptors and is 94% homologous to the human NPY-Y2 receptor [6,7], it is thought to be the mouse homologue of the NPY-Y2 receptor (Fig. 2). The cytoplasmic tail of this receptor contains five serine residues and three threonine residues, which are possible phosphate acceptors and which are also observed in the human NPY-Y2 receptor. Furthermore, there seems to be one N-linked glycosylation site in the N-terminal domain (Fig. 1). A long polyadenylation sequence was found at the

Fig. 1. The nucleotide sequence of cloned mY2 and the deduced amino-acid sequences for the encoded mouse NPY-Y2 receptor. The predicted amino-acid sequence is shown in single-letter codes under the nucleic acid sequence. The seven putative transmembrane domains are underlined. The '#' under an amino-acid sequence indicates a potential N-linked glycosylation site in the N-terminal extracellular region.

3′ end of the clone, suggesting that the cDNA clone encompasses most of the mRNA species observed in the Northern blot (Fig. 3). Comparison of the amino-acid sequence of the mouse Y2 receptor to those of the mouse Y1 and Y4 receptors reveals significant differences. As shown in Fig. 2, at the amino-acid level the mouse Y2 receptor exhibits low overall identities of 27% and 26% with the Y1 and Y4 receptors, respectively. The alignment scores for the transmembrane (TM) domains are 42% and 44% identity with the Y1 and Y4 receptors, respectively. Interestingly, a high identity was observed in the TM2, TM6 and TM7 domains of these receptors (52% with the Y1 receptor and 53% with the Y4 receptor). Southern blot analysis of the mouse genomic DNA revealed the presence of single *Bam*HI, *Pst*I, *Eco*RI, and *Eco*RV restriction fragments, when probed with the full-length cDNA of mY2 clone (Fig. 3A). This result indicates that there is a single Y2 receptor gene. The expression of the receptor mRNA in various mouse tissues was investigated by Northern blot analysis (Fig. 3B). Among the tissues, only brain showed a band in the range of about 4 kilobases. No signal was obtained from any of the other peripheral tissues, such as heart, spleen, lung, liver, skeletal muscle, kidney, or testis.

To examine signal transduction through the cloned receptor, the coding region of mY2 was subcloned in a mammalian expression vector (pcDNA3, Invitrogen) and transfected into CHO-K1 cells (termed CHO-mY2). In the CHO-mY2 cells, intracellular second messages were investigated by measuring ligand-evoked intracellular Ca^{2+} mobility and cAMP accumulation. As shown in Fig. 4A, fura-2 pentaacetoxymethyl ester (fura-2/AM)-loaded CHO-mY2 cells showed a dose-dependent increase in the intracellular Ca^{2+} concentration with the expected sensitivity to NPY (ED_{50} around 2 nM). The intracellular Ca^{2+} response was receptor-dependent, being elicited by 100 nM NPY, PYY, and NPY-(13–36) ($\Delta[Ca^{2+}]_i = 154 \pm 11$ nM, 162 ± 2 nM, and 152 ± 15 nM (mean ± S.D., $n = 3$), respectively), but not by 100 nM [Leu^{31},Pro^{34}]NPY or PP (Fig. 4B). Incubation of the CHO-mY2 cells with 20 μM forskolin produced cAMP (on average 12 pmol/$5 \cdot 10^4$ cells) over a 30 min period. In these cells, forskolin-

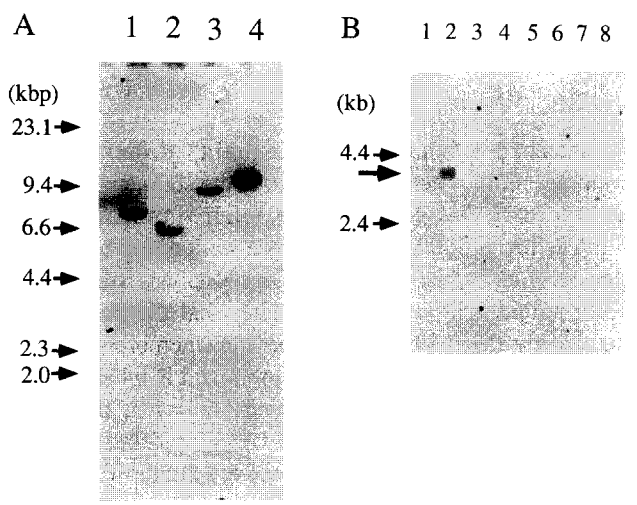


Fig. 3. Southern and Northern blot analyses. (A) Southern blot analysis of mouse genomic DNA. Mouse genomic DNA was purchased from Clontech. It was digested with *Bam*HI (lane 1), *Pst*I (lane 2), *Eco*RI (lane 3), or *Eco*RV (lane 4). The fragments were separated by electrophoresis on an 0.8% agarose gel, transferred onto a nylon membrane (Nybond-N$^+$, Amersham), and then hybridized with a ^{32}P-labeled full-length cDNA of mY2 as a probe. Hybridization was carried out at 65°C for 2 h in Rapid Hybridization Buffer (Amersham), and the filter was washed at 65°C for 30 min in 0.2×SSC containing 0.1% SDS. (B) Northern blot analysis of the mouse Y2 receptor mRNA. Mouse Multiple Tissue Northern Blot was purchased from Clontech. Each lane contained 2 μg of poly(A)$^+$ RNA. The full-length cDNA of mY2 was used as a probe. Hybridization was carried out at 65°C for 1 h in 1×SSC containing 0.1% SDS. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; and lane 8, testis.

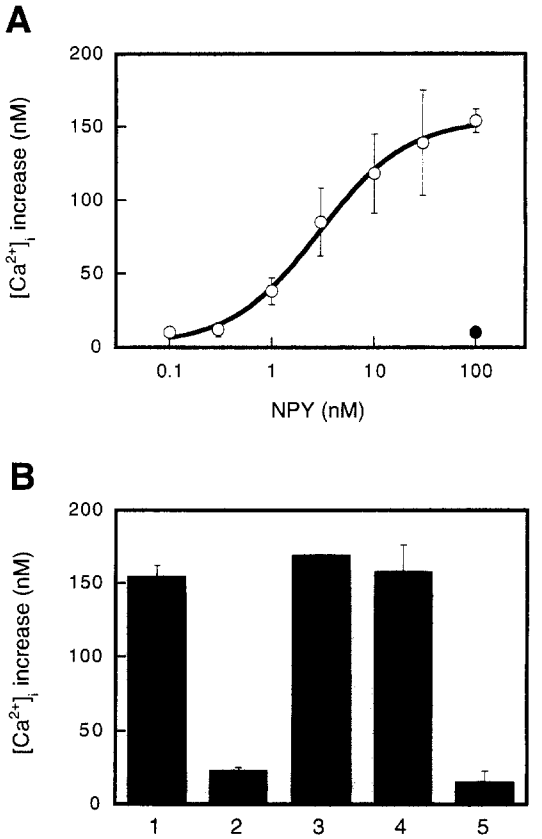


Fig. 4. Intracellular Ca^{2+} mobilization by NPY, NPY-(13–36), [Leu^{31},Pro^{34}]NPY, PYY, and PP in fura-2/AM-loaded CHO-mY2 cells. The cells were incubated in Hepes/Tyrode buffer containing 3 μM fura-2/AM for 1.5 h at room temperature. Measurements of the intracellular Ca^{2+} concentration in the fura-2/AM-load cells were performed as described previously [9]. (A) Dose–response curve of NPY for eliciting an intracellular Ca^{2+} increase in CHO-mY2 cells (○). To examine the effects of PTX, PTX (50 ng/ml) was added the medium 24 h before the assay. The NPY (100 nM)-induced response was inhibited by PTX (●). Each point is the mean ± S.D. of three assays. (B) Intracellular Ca^{2+} mobilization by pancreatic polypeptide family: lane 1, NPY (100 nM); lane 2, [Leu^{31},Pro^{34}]NPY (100 nM); lane 3, NPY-(13–36) (100 nM); lane 4, PYY (100 nM); and lane 5, PP (100 nM). The columns and vertical bars show the mean and S.D., respectively.

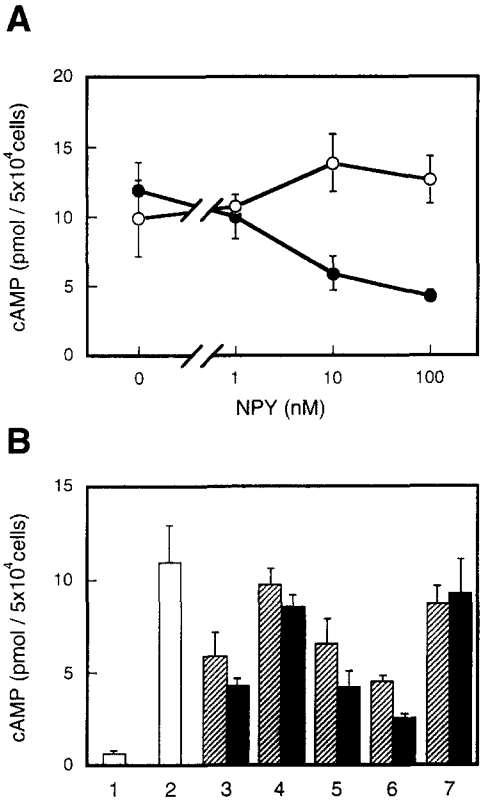


Fig. 5. Inhibition of forskolin (20 μM)-stimulated cAMP accumulation by pancreatic polypeptide family in CHO-mY2 cells. Measurements of the cAMP concentration in CHO-mY2 cells were performed using a cAMP Assay Kit (Amersham) as described previously [9]. (A) Forskolin-stimulated cAMP accumulation was inhibited by NPY in a dose-dependent manner (●). When CHO-mY2 cells were treated with PTX (50 ng/ml, 24 h), the inhibition of cAMP accumulation was eliminated (○). Each point is the mean ± S.D. of three assays. (B) Inhibition of forskolin-stimulated cAMP accumulation by pancreatic polypeptide family. Lane 1, vehicle; lane 2, forskolin; lane 3, forskolin plus NPY; lane 4, forskolin plus [Leu^{31},Pro^{34}]NPY; lane 5, forskolin plus NPY-(13–36); lane 6, forskolin plus PYY; and lane 7, forskolin plus PP (lanes 3–7, the hatched and filled columns show 10 nM and 100 nM peptides, respectively). The columns and vertical bars show the mean and S.D., respectively.

stimulated cAMP accumulation was inhibited by NPY in a dose-dependent manner (Fig. 5A). NPY did not inhibit the forskolin-stimulated accumulation of cAMP in CHO cells transfected with the pcDNA3 vector (data not shown). Upon the addition of 100 nM NPY, NPY-(13–36), or PYY to 5 CHO-mY2 cells, forskolin-stimulated cAMP accumulation was inhibited by 62 ± 3%, 63 ± 5% or 71 ± 2% (mean ± S.D., $n = 3$), respectively, but not by 100 nM [Leu^{31},Pro^{34}]NPY or PP (Fig. 5B). The increase in intracellular Ca^{2+} and the inhibition of forskolin-stimulated cAMP accumulation by NPY, PYY, and NPY-(13–36) were comparable to those observed with the human NPY-Y2 receptor [6,7]. These results suggest that the cloned mouse NPY-Y2 receptor is functionally expressed in CHO-mY2 cells. When CHO-mY2 cells were treated with PTX (50 ng/ml, 24 h), elevation of the intracellular Ca^{2+} concentration and inhibition of forskolin-stimulated cAMP accumulation by NPY were eliminated (Fig. 4A and Fig. 5A). This suggests that the mouse Y2 receptor couples to PTX-sensitive G-protein(s), probably Gi/Go, in CHO cells.

In summary, we have presented the structure and functional expression of the mouse NPY-Y2 receptor. The results of this investigation will be very useful for studying the tissue distribution of the NPY-Y2 receptor and for designing specific NPY agonists or antagonists for therapeutic purposes.

The authors wish to thank Miki Shibata, Sayaka Inoue, Mika Saitoh, Kiyoshi Ishii and Minoru Kumai of Japan Tobacco Life Science Research Laboratory for their outstanding technical assistance.

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