Identification of Two Isoforms of Mouse Neuropeptide Y-Y1 Receptor Generated by Alternative Splicing

ISOLATION, GENOMIC STRUCTURE, AND FUNCTIONAL EXPRESSION OF THE RECEPTORS*

(Received for publication, April 28, 1995, and in revised form, August 21, 1995)

Motonao Nakamura‡, Chie Sakanaka, Yoshiko Aoki‡, Hiroyuki Ogasawara, Takashi Tsujii, Hisashi Kodama, Takashi Matsumoto, Takao Shimizu, and Masana Noma‡

From the ‡Life Science Research Laboratory, Japan Tobacco Inc., 6-2 Umeoaka, Aoba-ku, Yokohama, Kanagawa 227, J. Japan and the ¶Department of Biochemistry, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, J. Japan

Two cDNA clones homologous with human neuropeptide (NP) Y-Y1 receptor have been isolated from a mouse bone marrow cDNA library. One was thought to be the cognate of the human NPY-Y1 receptor, termed Y1α receptor, and the other form, termed Y1β receptor, differed from the Y1α receptor in the seventh transmembrane domain and C-terminal tail. Analysis of the mouse genomic DNA showed that both receptors originated from a single gene. The different peptide sequences of the Y1β receptor were encoded by separate exons, hence, these receptors were generated by differential RNA splicing. High affinity binding of [125I]NPY to each receptor expressed in Chinese hamster ovary (CHO) cells and sequestration of [125I]NPY after binding to each receptor were observed. In the CHO cells expressing the Y1α receptor, intracellular Ca2+ increase, inhibition of forskolin-induced cAMP accumulation, and mitogen-activated protein kinase (MAPK) activation were observed by stimulation of NPY, and these responses were abolished by pretreatment with pertussis toxin. Since wortmannin completely inhibited NPY-elicited MAPK activation, we speculate that wortmannin-sensitive signaling molecule(s) such as phosphoinositide 3-kinase may lie between pertussis toxin-sensitive G-protein and MAPK. In contrast, these intracellular signals were not detected in CHO cells expressing the Y1β receptor. Northern blots and reverse transcriptase-polymerase chain reaction analyses indicated that the Y1α receptor was highly expressed in the brain, heart, kidney, spleen, skeletal muscle, and lung, whereas the Y1β receptor mRNA was not detected in these tissues. However, the Y1β receptor was expressed in mouse embryonic developmental stage (7 and 11 days), bone marrow cells and several hematopoietic cell lines. These results suggest that the Y1β receptor is an embryonic and a bone marrow form of the NPY-Y1 receptor, which decreases in the expression during development and differentiation.

Neuropeptide Y (NPY), a 36-amino acid peptide, is an important regulator in central and peripheral nervous systems (1). NPY is highly conserved in primary structure among species as sequences of human, rabbit, rat, and mouse are identical and differ from the porcine sequence by only a single amino acid (2). It belongs to a peptide family that also includes peptide YY (PYY) and prepropolypeptide (PP) (3). Mammalian NPY and PYY show 70% sequence identity, while PP is 50% homologous to NPY. NPY is widely distributed in the brain (4) and the peripheral nervous system (5), and is often co-localized with norepinephrine, e.g., in sympathetic perivascular nerve fibers (4, 5). Studies of various organs and cell types with peptide fragments of NPY have indicated that multiple NPY receptor subtypes exist (6). The two major receptor subtypes have been designated Y1 and Y2, and the Y1 receptor has the ability to respond to an analog of NPY modified at residues 31 and 34 ([Leu31, Pro34]NPY) (7). The Y2 receptor subtype is defined on the basis of its affinity to the NPY peptide C-terminal fragment NPY-(13-36) (8). More recently, data from several laboratories have indicated the existence of a Y3 type receptor to which PYY shows a markedly lower affinity than NPY (9). NPY receptors have been identified in a variety of tissues, including brain, spleen, small intestine, kidney, testis, and placenta (10–12). In addition, binding sites have been noted in human cell lines such as SK-N-MC cells (neuroblastoma cell line) and HEL cells (erythroleukemia cell line) (13, 14).

NPY mRNA and NPY-like immunoreactivity has been detected in rat megakaryocytes and platelets as well as in preparations of rat mononuclear blood cells (15). In particular, high levels of NPY mRNA and NPY-like immunoreactivity were found in bone marrow of certain species of autoimmune mice that develop B-cell lymphoproliferative disorders (15, 16) and in bone marrow and peripheral lymphoblasts of children with B-cell precursor leukemia (17). These results suggest that NPY may function in hematopoietic and/or immune systems, as well as in nervous systems.

Human (18, 19) and rat (20, 21) brain NPY receptor cDNAs were isolated and showed a sequence similar to members of a G-protein coupled receptor superfamily. Ligand-binding characteristics of the expressed protein showed that the cDNA encodes the Y1-type receptor. In addition, the rat Y1-type receptor was demonstrated not only in brain but also in splenic lymphocytes, by means of polymerase chain reaction (PCR) and long chain reaction; Fura-2/AM, Fura-2 pentaacetoxyethyl ester; BATA/AM, 1,2-bis-(o-amino phenoxo)-ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester; CHO cells, Chinese hamster ovary cells; PTX, pertussis toxin; MAPK, mitogen-activated protein kinase; G-protein, guanine nucleotide-binding protein; kb, kilobase pair(s); bp, base pair(s).
Characterization of Two Isoforms of the NPY-Y1 Receptor

3103

ligand-binding experiments (22). However, the signal transduction mechanism through the Y1-type receptor such as mitogen-activated protein kinase (MAPK) activation has not been clarified. We isolated the cognate mouse NPY-Y1 receptor cDNA from a bone marrow cell cDNA library and found a novel receptor form differing in seventh transmembrane and C-terminal tail domains. We describe here the origin of this structural diversity of the cloned mouse NPY-Y1 receptors, and provide novel information on the distribution of mRNAs encoding the two receptor isoforms, and on the cellular signaling of the cloned receptors expressed in Chinese hamster ovary (CHO) cells.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: [125I]NPY (81.4 TBq/mmol) and [α-32P]JdCTP (111 TBq/mmol) from DuPont NEN; human [Leu8,Pro8]NPY, human NPY (13-36), porcine PYY, and genetine from Sigma; human NPY from Peptide Institute, Inc. (Osaka); λZAP II vector from Stratagene; oligopent dT30-Super from Takara Shuzo (Kyoto); expression vector pcDNA/mneo from Invitrogen; forskolin and wortmannin from Seikagaku Kogyo (Tokyo); BAPTA/AM from Dojin (Kumamoto); pertussis toxin (PTX) from Funakoshi (Tokyo); U-73122 from BIOMOL Research Laboratory Inc.; antimouse monoclonal antibodies from Upstate Biotechnology Inc.; cDNAs of human brain, kidney, lung, vascular endothelial cells, smooth muscle, leukocytes, and bone marrow cells from Clontech; and various DNA-modifying enzymes from Takara Shuzo (Kyoto). All other chemicals were of analytical grade. Reagents for cell culture were from Nissui (Tokyo) and Life Technologies, Inc. WEHI-3 cells and L5178Y cells were obtained from the RIKEN Cell Bank (Tsukuba). ST2 cells were kindly provided by the JCRB Cell Bank (Tokyo).

PCR Analysis of the Human NPY-Y1 Receptor—To obtain the human NPY-Y1 receptor cDNA fragment, primers H1 (5′-GAATTCAACATTATTTTTCCAG-3′) and H2 (5′-TTGATCAAAGCACACGTATTTGTC-3′) were designed based upon published sequences (18, 19) and PCR was performed under the above conditions, using a template cDNA (1 mg) of human brain cDNA. The amplified DNA fragment (605 bp long), termed hY1, was subcloned into the mammalian expression vector pcDNA/mneo and the plasmid was transfected into CHO-K1 cells by electroporation. Stably transfected cells were selected in medium containing 1 mg/ml genetin (G418, Sigma) and cloned cell lines were obtained by limiting dilution. G418-resistant clones were expanded and tested for the ability to bind [125I]NPY. DNA PCR was performed using 1 μg of the synthesized cDNA with 30 cycles of 1 min of denaturation at 94 °C, 2 min of annealing at 57 °C, and 1 min of extension at 72 °C. The amplified products were separated by electrophoresis on an 0.8% agarose gel, transferred onto a nylon membrane (Hybond-N+), then hybridized with a 32P-labeled EcoRI-PstI fragment of the NPY-Y1 receptor. Hybridization was carried out at 65 °C for 2 h in Rapid Hybridization Buffer and the filter was washed at 65 °C for 30 min in 0.2 × SSC containing 0.1% SDS. RT-PCR Analysis—RT-PCR analysis was carried out using poly(A)+ RNA isolated from mouse bone marrow cells. The first-strand cDNA was synthesized using First-strand Synthesis Kit (Pharmacia). The forward oligonucleotide primers were derived from the common region to the Y1α and Y1β receptor (SP1: 5′-CCCCCTCTGACCCACAGGTC-3′ and SP2: 5′-AGATACATCCTCGTGGAAAGGAG-3′) and the reverse primers were derived from the unique regions of the Y1α receptor (aAP1, 5′-AAAAAGGCTTTTGACGACGTTG-3′; aAP2, 5′-CATGTTCTGCTGTCG-3′) and the Y1β receptor (bAP1, 5′-AGTACAGGCGACAG-3′ and bAP2, 5′-ATGGGATCATACAGGTTGAC-3′), respectively. PCR was performed using 1 μg of the synthesized cDNA with 30 cycles of 1 min of denaturation at 94 °C, 2 min of annealing at 57 °C, and 1 min of extension at 72 °C. The amplified products were separated by electrophoresis on an 0.8% agarose gel, transferred onto a nylon membrane (Hybond-N+), then hybridized with a 32P-labeled probe. The probe DNA fragment (170 bp long) was prepared by PCR reaction, using the following primers P1 (5′-ATGAGAAATCTCCGGGACAC-3′) and P2 (5′-AGCAGATTGGTTGCAAGGT-3′) and the Y1α receptor cDNA as a template, under the thermal cycling conditions described above. Hybridization was carried out at 65 °C for 2 h in Rapid Hybridization Buffer and the filter was washed at 65 °C for 30 min in 0.2 × SSC containing 0.1% SDS.

Stable Expression in CHO-K1 Cells—CHO-K1 cells were maintained in Ham's F-12 medium containing 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. For all experiments, cells were grown at 37 °C in a humidified atmosphere with 5% CO2. Each full-length cDNA fragment (Y1α or Y1β receptor) was subcloned into the mammalian expression vector pcDNA/mneo and the plasmid was transfected into CHO-K1 cells by electroporation. Stably transfected cells were selected in medium containing 1 mg/ml genetin (G418, Sigma) and cloned cell lines were obtained by limiting dilution. G418-resistant clones were expanded and tested for the ability to bind [125I]NPY. DNA PCR was performed using 1 μg of the synthesized cDNA with 30 cycles of 1 min of denaturation at 94 °C, 2 min of annealing at 57 °C, and 1 min of extension at 72 °C. The amplified products were separated by electrophoresis on an 0.8% agarose gel, transferred onto a nylon membrane (Hybond-N+), then hybridized with a 32P-labeled probe. The probe DNA fragment (170 bp long) was prepared by PCR reaction, using the following primers P1 (5′-ATGAGAAATCTCCGGGACAC-3′) and P2 (5′-AGCAGATTGGTTGCAAGGT-3′) and the Y1α receptor cDNA as a template, under the thermal cycling conditions described above. Hybridization was carried out at 65 °C for 2 h in Rapid Hybridization Buffer and the filter was washed at 65 °C for 30 min in 0.2 × SSC containing 0.1% SDS.

Binding Assay—Cell membranes of these transfected cells, prepared as described previously (25), were resuspended in ice-cold assay buffer I (50 mM Tris-HCl, pH 7.4, 2 mM CaCl2, 5 mM KCl, 120 mM NaCl, 1 mM MgCl2, 0.1% bacitracin). Each type of cell membranes was incubated with various concentrations (Scatchard plot analysis) or 1.0 nm (displacement experiments) of [125I]NPY in 0.2 ml of assay buffer II (50 mM Tris-HCl, pH 7.4, 2 mM CaCl2, 5 mM KCl, 120 mM NaCl, 1 mM MgCl2, 0.1% bovine serum albumin (Sigma), 0.1% bacitracin) at 25 °C for 1 h. The reaction was terminated by adding 2 ml of ice-cold assay buffer II, and the unbound radioactive component was removed by rapid filtration. The bound radioactivity was measured in a γ-counter (1470 WIZARD, Pharmacia). The protein concentration was determined by the method of Bradford (26) using bovine serum albumin as a standard.

Internalization of NPY in Transfected CHO Cells—Transfected cells (2 × 106 cells) were cultured in 12-well plates. [125I]NPY binding was measured on whole cell monolayers, as described (21). To determine internalized [125I]NPY, monolayers were treated for 3 min with 200 mM Gly-HCl, pH 3, 200 mM NaCl (subsequent to binding incubation) to remove [125I]NPY bound to the exterior surface of the cells, then the acid-resistant (internalized) radioactivity were measured as described (27–30).

Measurement of Intracellular Ca2+ Concentrations—The cells were incubated in HEPES/Tyrode buffer containing 3 μM Fura-2-pentaexcemethyl ester (Fura-2/AM) (Dojin) for 1 h at room temperature. Measurements of intracellular Ca2+ concentrations switched to a serum-free medium containing the indicated concentrations of NPY, 0.5 mM 3-isobutyl-1-methoxyxanthine, and 20 μM forskolin. After a 30-min incubation at 37 °C, the reaction was stopped and the cells lysed by the addition of the final 6% (w/v) trichloroacetic acid, then left at −80 °C. cAMP was measured using a cAMP Assay Kit (Amersham), according to the manufacturer’s protocol.

MAPK Assay—MAPK activity was measured as described (32), using myelin basic protein as a substrate.

Western Blotting—Cell lysates were run on an SDS-polyacrylamide
Characterization of Two Isoforms of the NPY-Y1 Receptor

A human NPY-Y1 receptor cDNA was cloned from a human brain cDNA library (18, 19). This receptor consists of 384 amino acids and has seven putative transmembrane domains, as noted in other members of the G-protein coupled receptors and is 93% homologous to the human NPY-Y1 receptor, it is thought to be the mouse NPY-Y1 receptor. The sequence was identical to the Y1 receptor. Sequences around the putative junctions are conserved in the mouse genome library, and the Y1 receptor is expressed not only in the brain, kidney, and lung, but also in bone marrow cells, whereas it was not observed in vascular endothelial cells, smooth muscle, and leukocytes (Fig. 1).

To determine the functional role of the NPY-Y1 receptor in bone marrow cells, we isolated and sequenced mouse NPY-Y1 receptors from a bone marrow cell cDNA library. Upon screening of approximately 5 x 10^6 plaques of the bone marrow cell cDNA library, using the human NPY-Y1 receptor fragment as a probe, we obtained 2 positive clones. One of the isolated clones, represented by Y1b receptor, has a 2279-bp insert DNA containing a 1146-bp open reading frame. 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 93% homologous to the human NPY-Y1 receptor, it is thought to be the mouse homologue of the NPY-Y1 receptor (Fig. 2a, a and b). The cytoplasmic tail of this receptor consists of several regions and 4 threonine residues, as possible phosphate acceptors, and as observed in the human NPY-Y1 receptor. Furthermore, there seem to be four N-linked glycosylation sites in the N-terminal domain and the extracellular loops.

We then characterized the other clone represented by Y1a receptor. The sequence was identical to the Y1a receptor from the 5'-untranslated region to the third extracellular region, but was completely different in the seventh transmembrane, the cytoplasmic tail, and 3'-untranslated region (Fig. 2a, a and c). Addition of different DNA fragments at position 980 (numbering from the first base of the coding sequence) of the Y1a receptor created another reading frame downstream from this junction, which extends the coding region to a new stop codon located 14 bp downstream. As a consequence, a 79-amino acid C-terminal fragment of the Y1a receptor was replaced with a new 4-amino acid fragment in the C-terminal end of the Y1b receptor. Thus, the Y1b receptor does not carry part of the seventh transmembrane and C-terminal tail (Fig. 2b). To confirm the existence of two forms of mouse NPY-Y1 receptor in mouse bone marrow cells, RT-PCR analysis was carried out with template cDNAs constructed with mRNAs of the bone marrow cells. As shown in Fig. 3, the amplified products corresponding to each clone were detected. Thus, mRNAs encoding two isoforms of mouse NPY-Y1 receptor exist in bone marrow cells.

Isolation and Characterization of Mouse NPY-Y1 Receptor Genomic Clones—To determine whether or not Y1a and Y1b receptors are produced from a single gene, genomic structure of the mouse NPY-Y1 receptor was characterized. First, Southern blot analysis of the mouse genomic DNA indicated the presence of single BamHI, PstI, HindIII, KpnI, and XhoI restriction fragments, when probe with the EcoRI-PstI fragment which corresponds to a part of the common region to both Y1a and Y1b receptor cDNAs (Fig. 4a). This means that the Y1a and Y1b receptors originated from a single gene.

Upon screening of approximately 1.5 x 10^6 plaques of a mouse genomic library with the above probe (the EcoRI-PstI fragment), we obtained 5 positive clones. One of the isolated clones (2MY1a2), approximately 23 kb, was analyzed in detail. Restriction mapping of this clone is shown in Fig. 4b. Partial sequence and Southern blot analysis revealed that this clone contains the entire Y1a receptor open reading frame, including the 5'- and 3'-untranslated regions (Fig. 2a, a and b, and Fig. 4b), but not the Y1b receptor-specific region (Fig. 2c). The mouse genomic library was thus rescreened using a DNA probe corresponding to the Y1b receptor-specific genomic DNA sequences: at positions 980 to 1300 (320 bases) of the Y1b receptor (Fig. 2a, c). Screening of approximately 1.5 x 10^6 plaques yielded 3 positive clones. One of the isolated clones (2MY1b11), approximately 13 kb, was extensively characterized. Restriction mapping, sequencing of the partial fragments that hybridized to the probe, and comparison with the other receptor cDNAs allowed for determination of the intron/exon structure of the NPY-Y1 receptor gene. As shown in Fig. 4b, the Y1a receptor has at least two introns. The first intron, approximately 6.4 kb long, was located at -147/-148 in the 5'-untranslated region of the Y1a receptor. Since the major transcriptional start points of the mouse NPY-Y1 receptor were determined at positions -167, -182, -238, -247, and -263 in the 5'-untranslated region (33), the first exon consists of about 20-120 bp. The second intron (108 bp long) is located just after the proposed fifth transmembrane domain at position 697. A similar organization (locations of first and second introns) was observed in the human NPY-Y1 receptor gene (34). The Y1b receptor is produced by RNA splicing of the third intron, more than 15 kb long, located downstream from position 908 of the Y1a receptor. Sequences around the putative junctions are shown in Fig. 4c. These sequences fit well with the proposed consensus sequences for RNA splicing. These results taken together indicate that the variation in C-terminal peptides could be produced by alternative splicing of mRNA from the single gene encoding the mouse NPY-Y1 receptor. The (TG)29 and (GC)10 sequences, alternating purine/pyrimidine repeat, and the potential left-handed Z-DNA-forming sequences (35-39), were found in tracts of up to 50 bp long in the Y1a receptor 3'-untranslated region (Fig. 2b). Although the function of these sequences is unknown, the potential Z-DNA-forming sequences in the third intron might play a substantial role for splicing of the Y1b receptor mRNA.

Characterization of the Cloned NPY-Y1 Receptors in CHO Cells—To examine ligand binding properties and signal transduction through each receptor, the coding region of each clone was subcloned in a mammalian expression vector and transfected into CHO-K1 cells (termed CHO-NPYα for the Y1a receptor and CHO-NPYβ for the Y1b receptor). [125I]NPY specif-
Characterization of Two Isoforms of the NPY-Y1 Receptor

Physically bound to membranes from the CHO-NPYα and the CHO-NPYβ cells. The results of Scatchard plot analysis of the specific [125I]NPY binding to each receptor showed that both receptors exhibited indistinguishable binding affinities with dissociation constants (Kd) of 1.0 ± 0.3 nM (mean ± S.D., n = 3) for Y1α receptor and 1.6 ± 0.5 nM (mean ± S.D., n = 3) for Y1β receptor. The pharmacological profiles of ligands competing for [125I]NPY binding to each receptor are consistent with that of a Y1-type receptor. The potency was: NPY > PYY > [Leu31,Pro34]NPY (Y1 agonist) >> NPY-(13–36) (Y2 agonist) (data not shown); similar rank orders of potency have been observed in human NPY-Y1 receptor-transfected cells (18, 19).

We also examined the kinetics of [125I]NPY internalization in transfected cells. The acid-NaCl wash is now a widely used method to determine location of the radioactive ligand bound to a cell surface (28, 29). The acid-resistant and acid-released radioactivity are then considered as the amount of internalized and cell surface-bound ligand, respectively. As shown in Fig. 5, total [125I]NPY binding to the CHO-NPYα cells and the CHO-NPYβ cells reached a maximum within 30 min at 37°C,
Figure 3. RT-PCR analysis of the two isoforms of NPY-Y1 receptor mRNAs in bone marrow cells. a, schematic presentation of PCR primers, corresponding positions in each cDNA and expected sizes of PCR products. b, gel electrophoresis of amplified products. Lanes 1-5 correspond to PCR analysis with a primer pair of SP2 and aAP2 in lane 1; SP1 and aAP1 in lane 2; SP2 and bAP2 in lane 3; SP2 and bAP1 in lane 4; SP1 and bAP1 in lane 5. c, Southern blot analysis of amplified products. After gel electrophoresis (panel b), DNA products were transferred onto nylon membrane and Southern blots made using an internal DNA fragment (prepared by PCR as described under “Experimental Procedures”) as a probe (represented by solid bar in a).

whereas internalization, defined as bound [125I]NPY to each cell not dissociable from the cells by acid (pH 3) treatment, was maximal (78.4% of total binding for the CHO-NPYα cells and 67.2% of total binding for the CHO-NPYβ cells) after 30 min, and negligible internalization occurred at 4°C (data not shown). These results suggest that NPY is internalized similarly after binding to each receptor.

To further determine the functional properties of each receptor, intracellular second messages were investigated by measuring NPY-evoked intracellular Ca2+ mobilization, cAMP accumulation, and MAPK activation in the transfected cells. Upon addition of NPY (10 nM) to the CHO-NPYα cells, intracellular Ca2+ was elevated (190 ± 28 nM, mean ± S.D., n = 5, Fig. 6A, a) and forskolin (20 μM)-stimulated cAMP accumulation was inhibited (49.5 ± 3.4% inhibition, mean ± S.D., n = 4). The intracellular Ca2+ response was receptor-dependent, being elicited by NPY, PYY, and [Leu31,Pro34]NPY, but not by NPY-(13-36) (data not shown). Elevation in intracellular Ca2+ by NPY, PYY, and [Leu31,Pro34]NPY was comparable to that observed with the human NPY-Y1 receptor (18). A transient generation of inositol trisphosphate evoked by NPY application, and NPY-induced intracellular Ca2+ increase was markedly suppressed by pretreatment with U-73122 (5 μM), a phospholipase C inhibitor (40, 41) (data not shown). When the CHO-NPYα cells were treated with PTX (50 ng/ml, 24 h), intracellular Ca2+ increase and inhibition of cAMP production were abolished (the former shown in Fig. 6A, b, and the latter not shown). This suggests that the Y1α receptor coupling to PTX-sensitive G-protein(s), probably Gi/Go. Several G-protein coupled receptors are reported to activate the signaling pathway involving MAPK activation (42). NPY at 10 nM activated MAPK in the CHO-NPYα cells (peaked at 3.5 min) (Fig. 6B). Since the NPY-elicited MAPK activation was completely abolished by PTX treatment (50 ng/ml, 24 h), the NPY receptor-mediated MAPK activation via PTX-sensitive G-protein(s) (Fig. 6C). Recently, we found that the platelet-activating factor-stimulated MAPK activation in guinea pig leukocytes is mediated by Ca2+-dependent and Ca2+-independent/wortmannin-sensitive pathways (43). To determine the pathways of MAPK activation by NPY in the CHO-NPYα cells, the effect of wortmannin was examined. Pretreatment of the cells with wortmannin inhibited MAPK activation in response to NPY, with a half-maximal inhibition observed at an inhibitor dose of 50-100 nM (data not shown). As shown in Fig. 6C, 500 nM wortmannin completely inhibited NPY (10 nM)-elicited MAPK activation. Treatment with wortmannin, at the concentration used in this experiment, did not affect intracellular Ca2+ mobilization (Fig. 6A, c). As shown in Fig. 6, A (panel d) and C, MAPK activity was not inhibited by the treatment of 20 μM BAPTA/AM (intracellular Ca2+ chelator), while it completely abolished the NPY-induced intracellular Ca2+ increase. These observations suggest that the Y1α receptor mediates MAPK activation via a Ca2+-independent but a wortmannin-sensitive pathway. Wortmannin-sensitive signaling molecules such as phosphoinositide 3-kinase may lie between MAPK activation and PTX-sensitive G-protein(s).

In contrast, NPY-elicited cell responses such as intracellular Ca2+ increase, inhibition of cAMP production, or MAPK activation was not detected in the CHO-NPYβ cells, although specific binding of [125I]NPY to the Y1β receptor was observed (Fig. 7). These results suggest that the cytoplasmic tail of the Y1α receptor contributes to G-protein(s) activation.

Expression of the Y1α and Y1β Receptor in Mouse Tissues—The relative abundance of mRNAs for the two receptors in various tissues was investigated by Northern blot analysis using differential DNA regions of the Y1α and Y1β receptor as probes. Fig. 8a indicates that the NPY-Y1α receptor mRNA, approximately 4.0 kb, was highly expressed in the brain, heart, kidney, spleen, skeletal muscle, and lung, whereas the mRNA of Y1β receptors was not detected in these tissues. Surprisingly, the Y1β receptor mRNA, approximately 4.5 kb, was highly expressed in the 7- and 11-day embryo (Fig. 8b). Since
Characterization of Two Isoforms of the NPY-Y1 Receptor

DISCUSSION

We report here the isolation of two isoforms of the NPY-Y1 receptor cDNA designated Y1α, a mouse homologue of the human NPY-Y1 receptor, and Y1β, a truncated form of Y1α (Fig. 2). RT-PCR, Southern blots, and genomic DNA analyses show that these two isoforms are generated from a single gene by alternative RNA splicing.

Despite the lack of seventh transmembrane domain and cytoplasmic tail, the Y1β receptor shows ligand binding specificities identical to those of the Y1α receptor. Walker et al. (44) reported that ionic interactions between the positively charged amino acids of NPY and negatively charged residues of the human NPY-Y1 receptor are involved in ligand-receptor interaction. By means of site-directed mutagenesis, substitution of acidic residues (aspartic acids and glutamic acids) present in the three extracellular loops of the human NPY-Y1 receptor yielded proteins unable to bind [125I]NPY. In contrast, deletion of the 51 residues in the C-terminal tail of the receptor resulted in a loss of 9 negatively charged residues but had no significant effect on affinity of the receptor for NPY. They suggested that extracellular loops of the NPY-Y1 receptor are involved in NPY binding, through ionic interaction. Since the Y1β receptor has a similar affinity to NPY, the extracellular loops rather than theseventh transmembrane domain plays a critical role for the ligand binding. Hunyady et al. (30) showed that PTX-sensitive G-proteins did not appear to play a role in endocytosis of angiotensin II (AT1a) receptor, since the receptor showed normal internalization kinetics in PTX-treated cells. They demonstrated that endocytosis of the AT1a receptor was independent of agonist-induced signal transduction, and receptor internalization and activation of phospholipase C led to different structural requirements of the receptor. Such independence of sequestration and the signal transduction was observed with the
37°C, the cells were challenged with 10 nM NPY, and cell lysates were starved for 20 h before stimulation. After prewarming for 10 min at p42 MAPK detected by immunoblot analysis. The cells were serum-B cells.

Characterization of Two Isoforms of the NPY-Y1 Receptor

We propose that the β-type receptor has a functional role for NPY internalization, since sequestration of [125I]NPY was observed in the β-type receptor expressed CHO cells after ligand-receptor binding. Intracellular sequestration of the NPY-receptor complex might provide a specific message to the cells.

Although the two isoforms show identical ligand affinities, there are differences in cell signaling properties. The Y1α receptor elicits a PTX-sensitive intracellular Ca2+ increase, inhibition of cAMP accumulation, and MAPK activation, whereas the Y1β receptor evokes no such responses. Irie et al. (46) reported that the C-terminal tail of prostaglandin-E2 subtype receptor was essential for activation of G-protein. They showed that the C-terminal tail-truncated prostaglandin-E2 subtype receptor retained the potential to form the agonist/receptor/Gi-protein ternary complex but failed to activate G-protein. Hence, we speculate that the C-terminal tail of NPY-Y1 receptor contributes to the activation of G-protein(s).

Production of multiple isoforms by alternative splicing has been noted in the rhodopsin-type receptor family, such as D2 dopaminergic receptor (47–49), prostaglandin-E2 subtype receptor (50, 51), thromboxane A2 receptor (52), metabotropic glutamate receptor (53), neurokinin-1 receptor (54), MCP-1 receptor (55), and somatostatin receptor (56). All these receptor isoforms differ only in the third cytoplasmic loop or C-terminal tail, and these isoforms show no properties of the Y1β receptor: truncated structure and defect of cell signaling.

In CHO-NPYα cells, NPY-induced MAPK activation via PTX-sensitive G-protein was seen to be mediated by Ca2+-independent/wortmannin-sensitive pathway. Several G-pro-
tein coupled receptors, including the α2-adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64), have been shown to stimulate MAPK activation in various cell types. The signaling pathways by which these receptors activate MAPK are poorly understood, but there are several pieces of evidence for both Ras-dependent (57–62) and Ras-independent (64–66) activation of MAPK. Koch et al. (67) reported that MAPK activation via PTX-sensitive pathway is mediated by the βγ subunit of G-protein and occurs as a result of Ras activation (67). Activated Ras can then act as a molecular switch causing Raf-1 activation, and subsequently leading to activation (67). Activated Ras can then act as a molecular switch causing Raf-1 activation, and subsequently leading to activation (67).

We have described here the cloning and the elucidation of the Y1 receptor. The Y1 receptor was specifically expressed in the bone marrow cells and several hematopoietic cell lines. In contrast, the Y1 receptor mRNA was detected in embryo (7 and 11 days), bone marrow cells, and several hematopoietic cell lines. Functional role of the Y1 receptor was established by establishing knock-out mice.

Northern blots and RT-PCR analyses showed tissue- and development-specific expression of two isoforms of receptor mRNAs. The Y1α receptor was specifically expressed in the brain, heart, kidney, spleen, skeletal muscle, lung, bone marrow cells, and several hematopoietic cell lines. In contrast, the Y1β receptor mRNA was detected in embryo (7 and 11 days), bone marrow cells, and several hematopoietic cell lines. The functional role of the Y1β receptor in embryonic development and hematopoietic system can be further examined by establishing knock-out mice.

NPY mRNA and NPY-like immunoreactivity is found not only in autonomic nervous systems and in the adrenal medulla but also in megakaryocytes/platelets and possibly mononuclear blood cells in rats. In humans, NPY is present in lymphocytes and monocytes (68). A high level of NPY was found in bone marrow of autoimmune mice with B-cell lymphoproliferative disorders and in children with B-cell precursor leukemia. These findings suggest a role for NPY during normal B-cell development and/or pathologic disorders of B-line cells. The existence of the NPY-Y1 type receptor in rat splenic lymphocytes was deduced from PCR evidence and from ligand-binding analyses (22). The presence of the Y1α and Y1β receptors in bone marrow cells suggests a role for these receptors in the hematopoietic system.

We have described here the cloning and the elucidation of genomic structure, signal transduction, and tissue distribution of two isoforms of the mouse NPY-Y1 receptor with alternatively spliced C-terminal regions. NPY-Y1 receptors form a novel repertoire of G-protein-coupled receptors that are divergent from other currently known G-protein-coupled receptors, including the α2-adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64). NPY-Y1 receptors form a novel repertoire of G-protein-coupled receptors that are divergent from other currently known G-protein-coupled receptors, including the α2-adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64). NPY-Y1 receptors form a novel repertoire of G-protein-coupled receptors that are divergent from other currently known G-protein-coupled receptors, including the α2-adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64). NPY-Y1 receptors form a novel repertoire of G-protein-coupled receptors that are divergent from other currently known G-protein-coupled receptors, including the α2-adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64). NPY-Y1 receptors form a novel repertoire of G-protein-coupled receptors that are divergent from other currently known G-protein-coupled receptors, including the α2-adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64). NPY-Y1 receptors form a novel repertoire of G-protein-coupled receptors that are divergent from other currently known G-protein-coupled receptors, including the α2-adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64). NPY-Y1 receptors form a novel repertoire of G-protein-coupled receptors that are divergent from other currently known G-protein-coupled receptors, including the α2-adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64). NPY-Y1 receptors form a novel repertoire of G-protein-coupled receptors that are divergent from other currently known G-protein-coupled receptors, including the α2-adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64). NPY-Y1 receptors form a novel repertoire of G-protein-coupled receptors that are divergent from other currently known G-protein-coupled receptors, including the α2-adrenergic receptor (57), lysophosphatidic acid receptor (58–60), M2 muscarinic acetylcholine receptor (61), C5a receptor (62), somatostatin receptor (32, 63), and platelet-activating factor receptor (64).
Characterization of Two Isoforms of the NPY-Y1 Receptor

Coughlin, S. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2752–2756
56. Vanetti, M., Vogt, G., and Hölt, V. (1993) FEBS Lett. 331, 260–266
57. Aldás, J., van Corven, E. J., Hordijk, P. L., Milligan, G., and Moolenaar, W. H. (1993) J. Biol. Chem. 268, 22235–22238
58. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L., and Moolenaar, W. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1257–1261
59. Howe, L. R., and Marshall, C. J. (1993) J. Biol. Chem. 268, 19196–19199
60. Buhl, A. M., Avdi, N., Worthen, G. S., and Johnson, G. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9190–9194
63. Bito, H., Mori, M., Sakanaka, C., Takano, T., Honda, Z., Gotoh, Y., Nishida, E., and Shimizu, T. (1994) J. Biol. Chem. 269, 12722–12730
64. Honda, Z., Takano, T., Gotoh, Y., Nishida, E., Ito, K., and Shimizu, T. (1994) J. Biol. Chem. 269, 2307–2315
65. Crews, C. M., and Erikson, R. L. (1993) Cell 74, 215–217
66. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993) Science 260, 315–319
67. Koch, W. J., Hawes, B. E., Allen, L. F., and Lefkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12706–12710
68. Schwarz, H., Villiger, P. M., Kempis, J. V., and Lotz, M. (1994) J. Neuroimmunol. 51, 53–61
Identification of Two Isoforms of Mouse Neuropeptide Y1 Receptor Generated by Alternative Splicing: ISOLATION, GENOMIC STRUCTURE, AND FUNCTIONAL EXPRESSION OF THE RECEPTORS

Motonao Nakamura, Chie Sakanaka, Yoshiko Aoki, Hiroyuki Ogasawara, Takashi Tsuji, Hisashi Kodama, Takashi Matsumoto, Takao Shimizu and Masana Noma

J. Biol. Chem. 1995, 270:30102-30110.
doi: 10.1074/jbc.270.50.30102

Access the most updated version of this article at http://www.jbc.org/content/270/50/30102

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 68 references, 35 of which can be accessed free at http://www.jbc.org/content/270/50/30102.full.html#ref-list-1