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ACCELERATED COMMUNICATION

Sequence and Expression of a Neuropeptide Y Receptor cDNA

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SUMMARY

The polymerase chain reaction was used to isolate novel GTP-binding protein (G protein)-coupled receptors from bovine locus coeruleus (LC), a brain region enriched in the neuropeptide Y (NPY) system, using degenerate primers derived from the third and sixth transmembrane domains of known G protein-coupled receptors. Partial sequence analysis revealed that the polymerase chain reaction cDNA fragments were homologous to other G protein-coupled receptors. One of these cDNA fragments was used to isolate a full length cDNA clone, referred to as LCR1, from an LC cDNA library. LCR1 is 1.7 kilobases in length and encodes a predicted protein of 353 amino acids, with a membrane topology similar to that of other G protein-coupled receptors. Expression of LCR1 in mammalian cells revealed saturable and specific high affinity binding for ^{125}I-NPY but not for any of the other ligands tested. Northern blot analysis revealed that labeled LCR1 DNA hybridized with a predominate mRNA transcript of approximately 1.7 kilobases, which was found to be most abundant in LC, cerebellum, and pons, intermediate in dorsal raphe, substantia nigra, and thalamus, and lowest in cerebral cortex and neostriatum. Significant levels of LCR1 mRNA were also present in heart, kidney, lung, and liver. This cDNA clone will be useful for studies of the regulation and function of NPY receptors, as well as for the isolation of related NPY receptor subtypes.

NPY is a member of the pancreatic polypeptide family that is widely distributed throughout the peripheral and central nervous systems (1–4). Members of this peptide family are 36 amino acids long and include PP, which, in contrast to NPY, is predominantly localized in endocrine cells of the gastrointestinal tract (5, 6).

In the peripheral nervous system, NPY is colocalized with norepinephrine in sympathetic neurons, where it inhibits prejunctional release of norepinephrine and directly influences a variety of postjunctional target tissues. Some of the actions of NPY in the periphery include regulation of vasoconstriction, smooth muscle contraction, cardiac function, and hormone secretion (2, 3, 7–9). In the central nervous system, NPY is colocalized with noradrenergic and adrenergic cell bodies in the brainstem, including the LC, and is also colocalized with γ-aminobutyric acid and somatostatin in cerebral cortex and elsewhere (3, 10–12). In fact, NPY is the most abundant identified neuropeptide in brain. NPY exerts a number of effects on the brain; it is an extremely potent stimulant of feeding behavior, regulates blood pressure, heart rate, and respiration, and influences hypothalamic neuroendocrine systems (13–20).

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NPY ligand binding sites have been characterized in both peripheral tissues and brain and, based on evidence from functional studies, NPY receptor subtypes referred to as Y_1 and Y_2 have been proposed (3, 21–24). Indirect evidence suggests that NPY receptors belong to the superfamily of G protein-coupled receptors (25, 26). NPY receptor activation inhibits cyclic AMP production in brain and cultured cells and inhibits sustained calcium currents in rat dorsal root ganglion neurons in culture (27–30). In addition, NPY receptor binding and function are sensitive to guanine nucleotides and inactivation of G proteins by pertussis toxin (23, 29, 31, 32).

In the present study, PCR was used to isolate an NPY receptor from bovine LC, a relatively homogeneous brainstem noradrenergic nucleus that is enriched in the NPY system (10–12). We now report on the sequence and expression of a cDNA for a novel G protein-coupled receptor that encodes an NPY receptor.

Experimental Procedures

PCR amplification of LC cDNA. The PCR was used to amplify double-stranded LC cDNA, using a pair of degenerate oligonucleotide primers. The forward primer consisted of a 27-mer with a degeneracy of 256, and the reverse primer was a 29-mer with a degeneracy of 64.

ABBREVIATIONS: NPY, neuropeptide Y; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; PP, pancreatic polypeptide; LC, locus coeruleus; G protein, GTP-binding protein; CRF, corticotropin-releasing factor; VIP, vasoactive intestinal peptide; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

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The sequences of the primers have been published and were derived from conserved regions of the third and sixth transmembrane domains of the β_1-, β_2-, and α_2-adrenergic receptors, the 5-hydroxytryptamine$_{1A}$ receptor, the substance K receptor, and the M_1 muscarinic receptor (33). Double-stranded cDNA was synthesized using the Amersham cDNA Synthesis System Plus, with oligo(dT) priming of poly(A)$^+$ RNA isolated from bovine LC. The cDNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended at 0.5 μg/μl in sterile Milli-Q water. LC cDNA (0.25 μg) was subjected to 35 rounds of PCR amplification in 100 μl, using a Coy temperature block cycler. The reaction mixture, containing 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM of each deoxynucleoside triphosphate, and 0.25 μM of each primer, was heated to 70° for 3 min and to 95° for 1.75 min and was cooled to 60°, and 2.5 units of AmpliTaq DNA polymerase (Cetus) were added. The PCR cycle profile was as follows: 1.75 min at 95°, 2.75 min at 46°, and 2.25 min at 70°. After the 35 rounds of amplification, a final incubation for 12 min at 72° was performed. Approximately nine DNA bands, ranging in size from 400 to 1500 base pairs, were amplified by this approach.

Cloning and isolation of PCR-amplified DNA. PCR-amplified DNA was subcloned for sequence analysis and synthesis of labeled cDNA probes for library screening. After inactivation of the AmpliTaq DNA polymerase by heating for 10 min at 99°, the total PCR-amplified DNA was incubated with 5 units of Klenow for 20 min at 22°, to make the DNA ends "blunt." The DNA was extracted with phenol/chloroform once and precipitated with ethanol twice, using 2 M ammonium acetate in order to remove the PCR primers and deoxynucleoside triphosphates (34). The modified PCR DNA was then ligated with approximately 140 ng of linearized pBluescript SK−, using 1 unit of T4 DNA ligase (Boehringer Mannheim) in 10 μl, and was incubated for 18–20 hr at 15° (34). Half of the ligation reaction was used to transform $CaCl_2$-competent XL-1 Blue bacteria (Stratagene), which were spread on Luria Broth plates, containing 50 μg/ml ampicillin, 100 mM isopropylthio-B-D-galactoside, and 2% Xgal, and were grown overnight at 37°. The insert size of clones was assayed by PCR amplification of DNA isolated from individual colonies; white colonies were transferred to 50 μl of 1% Triton X-100, 20 mM Tris, pH 8.5, 2 mM EDTA, and heated for 10 min at 95°. Bacterial debris was briefly spun down in an Eppendorf microfuge, and 5 μl of supernatant were transferred to a clean Eppendorf tube. The DNA was amplified by repeat PCR, using the same degenerate primers exactly as described above, and 10 μl were assayed on a 1% agarose Tris-borate/EDTA gel. Five independent clones, containing inserts from 400 to 550 base pairs, were identified and are referred to as PCR clones 1, 2, 3, 9, and 13. Double-stranded cDNA of the five PCR clones was sequenced by the Sanger dideoxy chain-termination protocol, using Sequenase 2.0 and [α-^{35}S]dATP, according to the manufacturer's directions (United States Biochemicals). The sequence of PCR clone 3 was 94% identical to the hamster β_2-adrenergic receptor, and PCR clone 13 was 92% identical to RDC7, which encodes an A1 adenosine receptor (33, 35). The other three clones have limited sequence homology with previously identified G protein-coupled receptors.

Screening of LC cDNA library. Approximately 1 × 10^6 plaques of a bovine LC cDNA library, constructed from oligo(dT)-primed poly(A)$^+$ RNA from bovine LC, inserted in λZAPII (Stratagene), and transferred to 40 duplicate 15-cm filters, were probed with 2 × 10^6 cpm/ml [α-^{32}P]dATP random-prime-labeled PCR-amplified DNA of PCR clones 1, 2, and 9, in HB-N (10 mM Tris-HCl, pH 7.4, 40% formamide, 4 × SSC, 10% dextran sulfate, 0.02% each of polyvinylpyrrolidone, bovine serum albumin, and Ficoll 400, and 100 μg/ml salmon sperm DNA), for 16 hr at 42° (36). The nitrocellulose filters were washed twice in 2× SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS, at room temperature and twice in 0.1× SSC, 0.1% SDS, at 65°. The filters were exposed to XAR film at −70° overnight. Sixteen positive plaques were isolated, five for PCR1, eight for PCR2, and three for PCR9. The cDNA clones inserted within the pBluescript phagemid were excised out of λZAPII, according to the Stratagene protocol. The phagemid was gel purified away from the R408 helper phage and used to transform $CaCl_2$-competent XL-1 Blue bacteria.

One of the cDNA clones for PCR1, which contained the longest insert, referred to as LCR1, was purified on a CsCl gradient and subjected to sequence analysis. The entire open reading frame of LCR1 was derived from a set of overlapping nested deletion fragments generated with Exonuclease III and mung bean nuclease, according to Stratagene's directions. These deletion clones were then sequenced by the Sanger dideoxy sequencing protocol, with Sequenase 2.0 and [α-^{35}S]dATP, according to the manufacturer's directions (United States Biochemicals). The sequence was verified by sequencing in the opposite direction.

Expression and transfection. LCR1 was subcloned into the *Hind*III and *Xba*I sites of the mammalian expression vector pcDNAI/Neo (Invitrogen), which contains a cytomegalovirus promoter, an SV40 origin of replication, and the neomycin resistance gene. This expression vector was used for transient and stable transfection, by lipofection, of COS-7 and CHO-K1 cells, respectively. COS cells were grown to 50–80% confluency in 15-cm dishes, in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamine, and 0.1 μg/ml gentamicin, and were transfected with LCR1-pcDNAI/Neo, using a 1:2.5 (w/w) mixture of dimethyldioctadecyl ammonium bromide and dioleoylphosphatidylethanolamine (Bethesda Research Laboratories); cells were harvested 48 hr later. Stable expression of LCR1 in CHO-K1 cells was achieved by lipofection using Transfectase, according to the manufacturer's directions (Bethesda Research Laboratories). LCR1-transfected cells were continuously selected with 400 μg/ml neomycin analogue G-418, in F12 medium. Northern blot analysis of mRNA isolated from transfected cells verified that LCR1 mRNA was expressed in both the COS and CHO cells.

Radioligand binding assays. Preliminary ligand binding assays were conducted on membranes isolated from transfected cells, and additional characterization of ^{125}I-NPY binding was carried out on intact stably transfected CHO cells. Particulate fractions were isolated from COS and CHO cells that had been rinsed in phosphate-buffered saline and scraped from culture plates. The cells were pelleted at 750 × g for 5 min and then homogenized with a Polytron (setting 6), in approximately 100 volumes of 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA. The homogenate was centrifuged at 48,000 × g for 15 min at 4°, and the particulate fraction was washed twice by resuspension of the pellet in the same buffer and centrifugation. These cells were resuspended in 50 mM Tris·HCl, pH 7.4, and analyzed for binding with a variety of radioligands, using standard conditions (carried out by NOVA Screen; NOVA Pharmaceutical, Baltimore, MD). The cells expressing LCR1 demonstrated specific binding of ^{125}I-NPY but not any of the other ligands tested. Regulation of ^{125}I-NPY binding by Gpp(NH)p was also examined in particulate fractions isolated from transfected CHO cells, under the same ligand-binding conditions.

^{125}I-NPY ligand binding was further characterized on intact stably transfected CHO cells. LCR1-transfected CHO cells were grown to confluency on microtiter plates, rinsed in Dulbecco's modified Eagle's medium (minus serum), and then incubated with various concentrations of ^{125}I-NPY (2200 Ci/mmol; New England Nuclear), in the presence or absence of competing unlabeled NPY (1 μM). Binding was carried out at 37° for 1 hr. For displacement experiments, 0.2–0.5 nM ^{125}I-NPY was used. The unbound ligand was removed by rinsing of the cells with culture medium. The cells were then solublized, and the remaining ^{125}I-NPY was quantitated by γ counting.

Northern blot analysis of mRNA. Total RNA was isolated from bovine tissues by the guanidinium isothiocyanate procedure and cesium chloride centrifugation (36). Poly(A)$^+$-enriched RNA was fractionated on denaturing formaldehyde-agarose gels and transferred to nitrocellulose for hybridization blot analysis, as described previously (36). Two DNA probes, labeled by random-primed synthesis using [^{32}P]dATP, were used for hybridizations; the first was a 450-base pair fragment of LCR1 generated by PCR using the same primers described above and

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the second was a 910-base pair *Hind*III-*Sma*I fragment of LCR1. Both probes yielded similar results. Nitrocellulose filters were hybridized with ^{32}P-labeled DNA for 18 hr at 42°, in HB-N. The filters were sequentially washed in 2× SSC, 0.1% SDS, at 25° and in 0.1× SSC, 0.1% SDS, at 55° and were then exposed to film for 48 hr without an intensifying screen.

Results and Discussion

mRNA from bovine LC was used to prepare double-stranded cDNA for the PCR reaction. Degenerate oligonucleotides derived from the sequence of the third and sixth transmembrane domains of related G protein-coupled receptors were prepared as primers for PCR amplification (33). This approach yielded nine major amplified DNA bands, ranging in size from 400 to 1500 base pairs; these bands could be derived from cDNAs for one of many receptors known to be enriched in LC, including NPY, CRF, VIP, opiate (μ and κ), and $GABA_B$ receptors, as well as various serotonergic, noradrenergic, and purinergic receptor subtypes (37). The entire PCR-amplified DNA was then cloned into pBluescript SK−. The insert size of the resulting clones was determined by subjecting DNA isolated from individual colonies to PCR and examining the amplified DNA bands on agarose gels. Five independent clones were isolated, ranging in size from 400 to 550 base pairs. Partial sequencing of these clones revealed that one was the bovine homologue of the β_2-adrenergic receptor and another was the bovine homologue of RDC7, which encodes an A1 adenosine receptor (33, 35). The other three have limited derived amino acid homology to other previously isolated receptors and, therefore, represent novel members of the G protein-coupled receptor family.

The PCR clones were used as probes to screen 1×10^6 plaques of an LC cDNA library constructed in λZAPII. Five cDNA clones were isolated for PCR clone 1. The clone containing the longest insert, referred to as LCR1, was further characterized. The sequence of LCR1, shown in Fig. 1, was derived from a set of overlapping nested deletion fragments and was confirmed by sequencing the fragments in the opposite direction. LCR1 has an ATG translation initiation codon, which results in an open reading frame of 353 amino acids. Analysis of the deduced amino acid sequence demonstrates the presence of seven putative hydrophobic transmembrane domains, a defining characteristic of G protein-coupled receptors (Fig. 2). The amino acid sequence of LCR1 has limited homology with other members of this receptor family, with 25 and 29% overall identity to the bovine substance K and the rat substance P receptors, respectively (e.g., see Fig. 2) (38, 39).

To determine the functional binding properties of LCR1, the cDNA insert was subcloned into the mammalian expression vector pcDNAI/Neo. This construct was used for transient expression of LCR1 in COS-7 cells and for stable expression in CHO-K1 cells, the latter achieved by continuously selecting cells with G-418, a neomycin analogue. High levels of LCR1 mRNA, analyzed by Northern blot analysis, were expressed in cells transfected with expression vector containing the insert but not in untransfected cells (data not shown). Particulate fractions were prepared from transfected cells and subjected to receptor binding analysis for a variety of neurotransmitter/neuropeptide ligands (carried out by NOVA Screen; NOVA Pharmaceuticals). The membranes from LCR1-transfected cells, both COS and CHO, displayed specific binding of ^{125}I-NPY but not any of the other ligands examined. Little or no

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-60   AAGTGACCCTGAGGACTTGAGTAGCCGGTAGCCCCCGCGGCTGGAGAGCAAGCGGTTACC

  1   ATGGAAGGGATCCGTATATTCACTTCCGATAATTACACCGAGGATGACTTGGGCTCGGGT
  1    M  E  G  I  R  I  F  T  S  D  N  Y  T  E  D  D  L  G  S  G
                                     ▲
 61   GACTATGATTCCATGAAGGAACCCTGCTTCCGGGAAGAAAATGCCCATTTCAACCGGATC
 21    D  Y  D  S  M  K  E  P  C  F  R  E  E  N  A  H  F  N  R  I

121   TTCCTGCCCACTGTCTACTCCATCATCTTCCTGACTGGCATAGTGGGCAACGGATTGGTC
 41    F  L  P  T  V  Y  S  I  I  F  L  T  G  I  V  G  N  G  L  V

181   ATTCTGGTCATGGGTTACCAGAAGAAACTAAGAAGCATGACGGACAAGTACAGACTGCAC
 61    I  L  V  M  G  Y  Q  K  K  L  R  S  M  T  D  K  Y  R  L  H

241   CTGTCTGTGGCGGACCTCCTGTTTGTCCTCACGCTTCCCTTCTGGGCAGTCGATGCTGTG
 81    L  S  V  A  D  L  L  F  V  L  T  L  P  F  W  A  V  D  A  V

300   GCAAACTGGTACTTTGGGAAGTTCCTCTGCAAGGCAGTCCATGTCATCTACACAGTCAAC
101    A  N  W  Y  F  G  K  F  L  C  K  A  V  H  V  I  Y  T  V  N

360   CTCTACAGCAGTGTCCTCATCCTGGCCTTTATCAGTCTGGACCGGTACCTGGCTATCGTC
121    L  Y  S  S  V  L  I  L  A  F  I  S  L  D  R  Y  L  A  I  V

420   CATGCTACCAACAGTCAGAAGCCAAGGAAGCTGCTGGCTGAAAAGGTGGTCTATGTTGGT
141    H  A  T  N  S  Q  K  P  R  K  L  L  A  E  K  V  V  Y  V  G

480   GTCTGGCTACCTGCTGTCCTGTTGACTATTCCTGATCTCATCTTTGCTGACATCAAGGAG
161    V  W  L  P  A  V  L  L  T  I  P  D  L  I  F  A  D  I  K  E

540   GTGGATGAGAGGTACATCTGTGATCGCTTCTATCCCAGCGACCTGTGGCTAGTGGTGTTT
181    V  D  E  R  Y  I  C  D  R  F  Y  P  S  D  L  W  L  V  V  F

600   CAGTTTCAGCACATCGTGGTCGGCCTTCTCCTGCCTGGTATCGTCATCCTGTCCTGCTAC
201    Q  F  Q  H  I  V  V  G  L  L  L  P  G  I  V  I  L  S  C  Y

661   TGCATTATCATCTCCAAGCTGTCCCACTCCAAAGGCTATCAGAAGCGCAAGGCCCTCAAG
221    C  I  I  I  S  K  L  S  H  S  K  G  Y  Q  K  R  K  A  L  K

721   ACCACAGTTATCCTCATCCTGACTTTCTTCGCCTGCTGGCTGCCCTACTACATTGGGATC
241    T  T  V  I  L  I  L  T  F  F  A  C  W  L  P  Y  Y  I  G  I

781   AGCATCGACTCCTTCATCCTTCTGGAAATCATCCAGCAAGGGTGTGAGTTTGAGAGCACT
261    S  I  D  S  F  I  L  L  E  I  I  Q  Q  G  C  E  F  E  S  T

841   GTGCACAAGTGGATTTCCATCACCGAGGCCCTAGCTTTTTTTCACTGCTGCCTGAATCCC
281    V  H  K  W  I  S  I  T  E  A  L  A  F  F  H  C  C  L  N  P

901   ATCCTCTATGCCTTCCTTGGGGCCAAATTTAAAACCTCTGCCCAGCATGCACTCACCTCT
301    I  L  Y  A  F  L  G  A  K  F  K  T  S  A  Q  H  A  L  T  S

961   GTGAGCAGAGGGTCCAGCCTGAAGATCCTCTCCAAGGGCAAGCGAGGTGGACATTCTTCT
321    V  S  R  G  S  S  L  K  I  L  S  K  G  K  R  G  G  H  S  S

1021  GTCTCAACCGAGTCTGAATCTTCAAGTTTTCACTCCAGCTAACACTGGACTTTTGTACAT
341    V  S  T  E  S  E  S  S  S  F  H  S  S  *

1081  TAAAGAACTTTTTTTTGAAGTTACACATTTTTTCAGATGTAAAAGACTGACCAACACTGT

1141  ACAGTTTTTATTGACTGTTGGATTTTTTTCTTGTGTTTGTTTAGTTTTTGTGAGGTTTAA

1201  TTGACTTATTTATATAAATA
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Fig. 1. Nucleotide and deduced amino acid sequences of a bovine NPY receptor cDNA. The first nucleotide and amino acid residue of the translation start site are designated as position 1. Putative hydrophobic transmembrane domains are *underlined*. Potential glycosylation site is noted with an *arrowhead*, and the stop codon is marked with a *star*.

specific binding was observed in membranes from untransfected COS or CHO cells. Additional ligand binding studies were carried out in cultures of intact, stably transfected, CHO cells. LCR1-transfected cells displayed a K_d of 1.3 nM and B_{max} of 64 fmol/mg of total cellular protein for ^{125}I-NPY (Fig. 3A). NPY displaced radiolabeled NPY with a K_i value of 1.5 nM (Fig. 3B; Table 1). In contrast, PP did not significantly displace NPY ligand binding in the transfected cells (Table 1). These affinity and displacement constants for NPY and PP are consistent with values reported for NPY ligand binding in brain membranes and cultured cells (21–23, 29, 40). NPY13-36 displayed a slightly higher potency to displace ^{125}I-NPY than [Leu^{31},Pro^{34}]NPY, with K_i values of 40 and 52 nM, respectively (Fig. 3B; Table 1). CRF and somatostatin, two unrelated neuropeptides, displaced ^{125}I-NPY binding, but with at least 2 orders of magnitude lower affinity, compared with NPY (Table 1). Other neuropeptides and neurotransmitters tested caused

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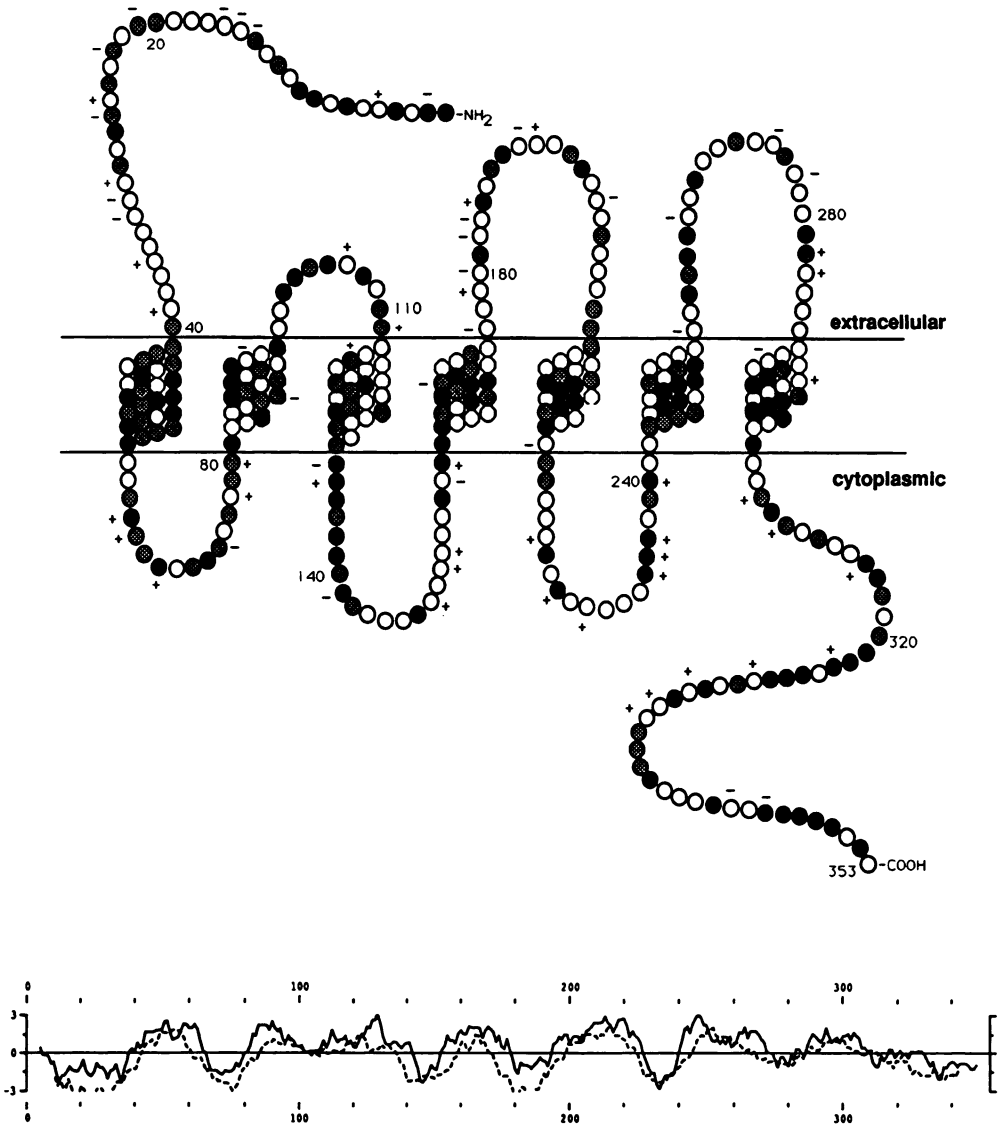

Fig. 2. Transmembrane model of the LCR1 NPY receptor and hydropathicity plot. *Upper*, the *solid* or *shaded circles* represent amino acids that are identical to or conserved substitutions of, respectively, the rat substance P receptor (39). Positively and negatively charged amino acids are also denoted. The transmembrane-spanning domains were identified by hydropathy analysis and homology to other G protein-coupled receptors. *Lower*, hydropathicity plot generated using the PEPPLOT program. Positive regions are relatively hydrophobic and negative regions are more hydrophilic. Putative transmembrane regions are numbered sequentially. *Solid* and *dashed lines* were generated using the Kyte-Doolittle and Goldman criteria, respectively.

no detectable displacement of ^{125}I-NPY binding at up to micromolar concentrations (Table 1). Finally, Gpp(NH)p dose-dependently decreased ^{125}I-NPY specific binding to particulate fractions isolated from transfected CHO cells, demonstrating that LCR1 encodes a G protein-coupled receptor (Fig. 4).

The distribution of LCR1 mRNA was examined by Northern blot analysis of bovine tissues. Labeled LCR1 hybridized to a predominant mRNA band of approximately 1.7 kb, which was most abundant in LC, cerebellum, pons, and kidney (Fig. 5). Moderate levels of hybridization were seen in dorsal raphe, substantia nigra, and thalamus, whereas lowest levels of LCR1 mRNA were observed in cerebral cortex and neostriatum. The same size mRNA band was also detected in rat tissues, with significant levels of mRNA being observed in cerebellum and several peripheral organs, including kidney, liver, lung, and heart (data not shown).

In this study, we present the sequence and expression of an NPY receptor cDNA clone isolated from bovine LC. This receptor is a member of the G protein-coupled receptor superfamily and displays specific and high affinity binding for ^{125}I-NPY. NPY receptors have been subdivided into Y_1 and Y_2 subtypes, with a tentative assignment to postjunctional and prejunctional sites, respectively (3, 23, 24). It is reasonable to assume that noradrenergic neurons of the LC, which coexpress NPY, express a prejunctional NPY receptor, which has been shown to regulate norepinephrine release and cell firing rate (41). However, LC neurons receive input from adrenergic neurons that contain NPY and, therefore, may also express postjunctional NPY receptors (10–12, 37). In addition, the ligand-binding characteristics of NPY receptors located in the bovine LC have not been determined. The Y_2 selective NPY analogue NPY13-36 displays a slightly higher potency to displace ^{125}I-

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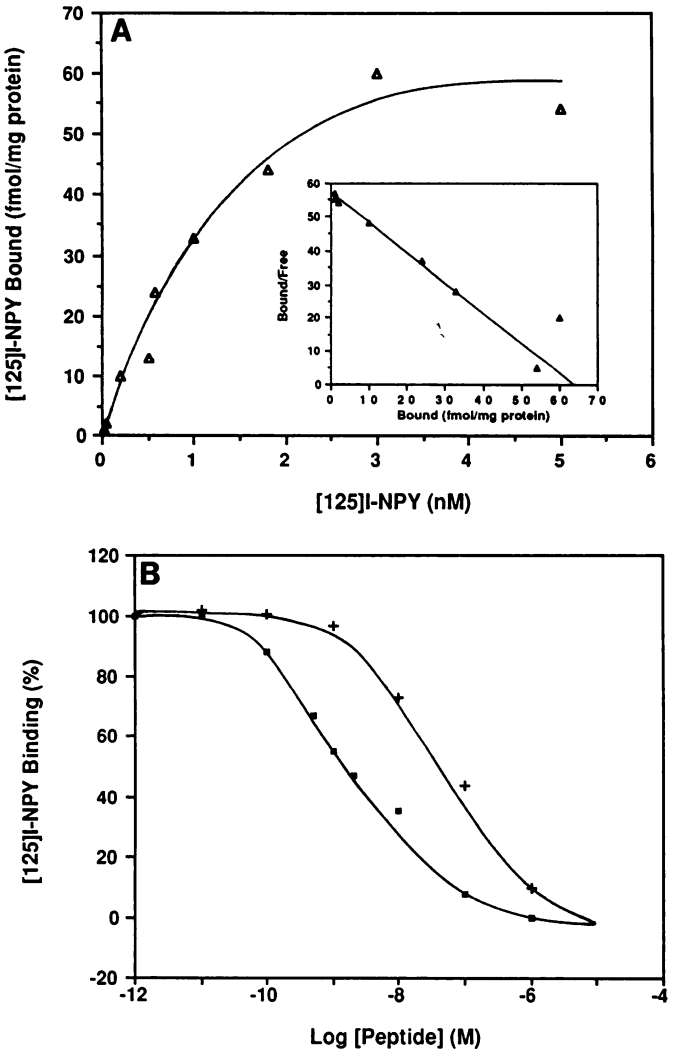

Fig. 3. ^{125}I-NPY binding to mammalian cells transfected with LCR1. A, Saturation isotherm of the specific binding of ^{125}I-NPY to LCR1-transfected CHO cells and Scatchard transformation of the saturation data (*inset*); the K_d and B_{max} were 1.3 nM and 64 fmol/mg of total cellular protein, respectively. B, Displacement of ^{125}I-NPY by unlabeled NPY (■) and pNPY13–36 (+). LCR1 was subcloned into the *Hind*III and *Xba*I sites of the mammalian expression vector pcDNAI/Neo and then transfected into CHO cells by lipofection, as described in Experimental Procedures. Stable LCR1 transfectants were made by selection of cells with G-418 (400 μg/ml). LCR1-transfected CHO cells were grown to confluency on microtiter plates, rinsed, and then incubated with various concentrations of ^{125}I-NPY, in the presence or absence of competing unlabeled NPY (1 μM). Binding was carried out at 37° for 1 hr. For displacement experiments, approximately 0.2–0.5 nM ^{125}I-NPY was used. The unbound ligand was removed by rinsing of the cells with culture medium. The cells were then solubilized, and the remaining ^{125}I-NPY was quantitated by γ counting. The *curves* presented are from one experiment and are representative of results of three separate experiments.

TABLE 1

Displacement of ^{125}I-NPY binding in LCR1-transfected cells

^{125}I-NPY binding in LCRI-transfected CHO-K1 cells was conducted as described for Fig. 2. Displacement constants were derived from the relationship $K_i = IC_{50}/1 + (F/K_d)$, where F is the concentration (nM) of radioligand added in each experiment and K_d is the affinity constant (1.3 nM) derived from ^{125}I-NPY saturation binding studies. Each value is the mean of two or three separate determinations.

Displacing agent	K_i
	nM
NPY	2.5
NPY 13–36	40
[Leu^{31},Pro^{34}]NPY	52
CRF	312
Somatostatin	985
PP	>1000
Vasopressin	>1000
VIP	>1000
Substance P	>1000
Endothelin	>1000
Norepinephrine	>1000
Serotonin	>1000
Acetylcholine	>1000

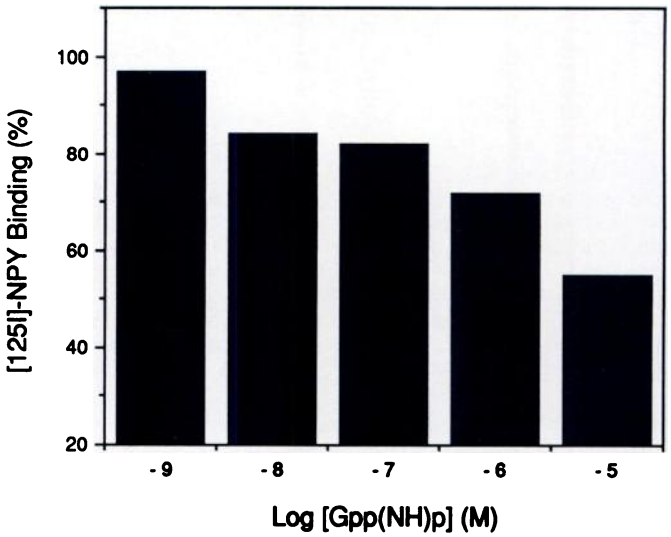

Fig. 4. Regulation of ^{125}I-NPY ligand binding to LCR1 by Gpp(NH)p. Particulate fractions were isolated from LCR1-CHO stable transfectants, as described in Experimental Procedures. ^{125}I-NPY ligand binding was carried out on a washed particulate fraction from LCR1-transfected cells, in the presence of various concentrations of Gpp(NH)p (10^{-9} to 10^{-5} M). Specific binding was determined in the presence of 1 μM NPY. The assay was conducted at 37° for 1 hr. Gpp(NH)p decreased specific ^{125}I-NPY binding but did not significantly influence nonspecific binding to LCR1. The results are expressed as percentage of specific binding and are the average of two separate experiments, each carried out in duplicate.

NPY binding to LCR1 than the Y_1 selective analogue [Leu^{31},Pro^{34}]NPY, and Northern blot analysis demonstrates a high level of LCR1 mRNA in LC. However, the presence of high levels of LCR1 mRNA in other brain regions and in peripheral tissues indicates that this NPY receptor is expressed in cell types other than noradrenergic neurons. The exact regional distributions of the Y_1 and Y_2 receptor subtypes in brain have not been established (40, 42). Therefore, the discrepancies between the regional distribution of LCR1 mRNA and that of total NPY ligand binding sites (e.g., cerebral cortex is low in LCR1 mRNA but high in NPY binding) are difficult to understand. Thus, the receptor encoded by LCR1 does not appear to have characteristics of the NPY receptor subtypes defined to date, and future studies will be needed to define its anatomical, pharmacological, and functional properties.

In the peripheral and central nervous systems, NPY is involved in the regulation of a variety of target tissues and neuronal systems and has been implicated in a number of medical conditions, most notably cardiovascular dysfunction (see Refs. 2–4). NPY has also been implicated in psychiatric disorders, particularly depression, anxiety, and eating disorders (3, 43, 44). The isolation and characterization of LCR1, and eventually of other NPY receptor subtypes, will contribute to a better understanding of the role played by the NPY system

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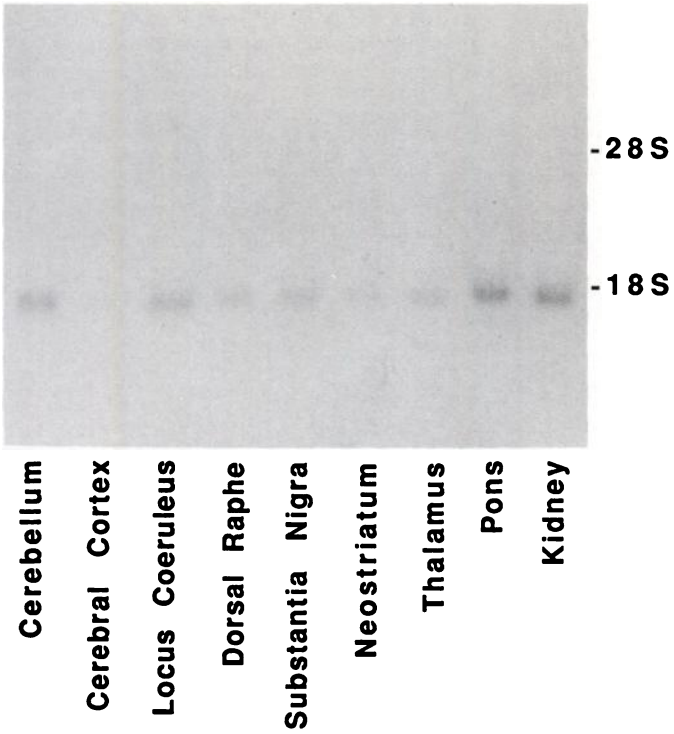

Fig. 5. Distribution of LCR1 mRNA. Poly(A)$^+$-enriched RNA (4 μg, except pons and kidney, which were 6 μg) was fractionated on denaturing formaldehyde-agarose gels and transferred to nitrocellulose for hybridization blot analysis, as described in Experimental Procedures. A 450-base pair DNA fragment of LCR1, corresponding to the third through sixth transmembrane domains, was labeled by random-primed synthesis, using [^{32}P]ATP. The nitrocellulose filters were hybridized and washed at high stringency, and resulting filters were exposed to film for 48 hr without an intensifying screen.

in normal physiological function. Moreover, expression of specific NPY receptor subtypes in cultured cells will aid in the development of novel pharmacological agents, which could prove useful for the treatment of a variety of abnormalities affecting peripheral tissues and brain.

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Note Added in Proof

Preliminary data indicate that peptide YY (PYY), which is a high affinity ligand for the Y_1 and Y_2 NPY receptor binding sites, displays a very low affinity ($K_i > 1$ μM) for LCR1. This provides further evidence that LCR1 is distinct from Y_1 and Y_2 and may represent a Y_3 NPY receptor subtype.

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