Molecular cloning and characterization of a new receptor for galanin

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Abstract Galanin (GAL) is a widely distributed neuropeptide with diverse biological effects including modulation of hormone release, antinociception and modification of feeding behavior. Its effects are mediated through G-protein-coupled receptors (GPCR) for which only a single type has been cloned, GAL receptor 1 (GALR1). We describe the cloning of a second galanin receptor type, GALR2, from rat hypothalamus. The GALR2 amino acid sequence is 38% identical to GALR1 and is pharmacologically similar to GALR1 when expressed in COS-7 cells. GALR2 is encoded by a single gene containing at least one intron and expressed in a diverse range of tissues.

Key words: Galanin; G-protein-coupled receptor; Feeding; Antinociception

1. Introduction

Galanin (GAL) is a widely distributed neuropeptide of 29 or 30 amino acids with a broad spectrum of biological effects, which include altering hormone and neurotransmitter release, antinociception, smooth muscle contraction and relaxation and feeding behavior [1]. Interest in GAL has recently peaked because of its reported effects on feeding behavior. Hypothalamic injection of GAL produces a dose-dependent increase in feeding in satiated rats which can be blocked by peptide receptor antagonists and antisense oligonucleotides to GAL [2–5]. Thus, the development of GAL receptor antagonists may be useful in the treatment of obesity [3,6].

Originally isolated from porcine intestine in a search for C-terminally amidated peptides [7], GAL has been found in humans and a wide range of species, with strict conservation of its primary sequence, particularly the first 15 residues [8]. As observed for most neuropeptides, GAL is synthesized as a part of a polyprotein precursor with canonical dibasic cleavage sites flanking mature GAL [9]. Studies of *GAL* gene expression localize its mRNA and protein to a variety of tissues [1,8–10], including the hypothalamus, hippocampus, locus coeruleus, anterior pituitary, spinal cord, pancreas and gastrointestinal tract.

The biological effects of GAL are mediated by G-protein-coupled receptors (GPCR) found at the cell surface of target cells which couple via pertussis toxin-sensitive G-proteins to downstream effector systems, including adenylate cyclase, phospholipase C and receptor-activated ion channels [1,11–14]. Galanin receptor cDNAs [15–17] from human melanoma cells and rat brain (and insulinoma cells) have been reported. GALR1 exhibits structural features consistent with GPCRs and is highly conserved between species. As predicted, GALR1 transiently expressed in CHO cells, couples in a negative fashion to adenylate cyclase [15,16]. GALR1 mRNA is prominently expressed in the hypothalamus, hippocampus, and spinal cord [15–18].

Distinct GAL receptor subtypes have been proposed [19,20] to account for the differential efficacy of GAL peptides to mimic GAL's actions and the variation in the ability of available chimeric peptides to serve as antagonists. We have used a low-stringency hybridization approach to identify and characterize a new member of the GAL receptor family, which we refer to as GALR2.

2. Materials and methods

2.1. Isolation and sequencing of galanin receptor cDNAs

A cDNA library from rat hypothalamus was constructed in the plasmid-based mammalian vector pcDNA-3 (InVitrogen, San Diego, CA). Total RNA was isolated from freshly dissected rat hypothalami (flash-frozen in liquid nitrogen) using the RNAgents total RNA isolation kit (Promega Biotech, Madison, WI) with a yield of approximately 0.5 mg from 1 g (wet weight) of hypothalamic tissue. Poly(A)$^+$ mRNA was selected using the Poly A tract mRNA Isolation System III (Promega Biotech) with a yield of approximately 6 μg from 0.5 mg total RNA. Three micrograms of poly(A)$^+$ was then utilized as a template for cDNA synthesis using a kit (Choice Superscript, Life Technologies, Gaithersberg, MD) with both random hexamer and oligo (dT)-*Not*I priming. The double-stranded cDNA was adapted for insertion into the *Bst*XI site of pCDNA-3 using *Eco*RI/*Bst*XI adapters and transformed by electroporation into the *Escherichia coli* strain HB101. The resulting library contained approximately 750 000 primary transformants with 90% of the clones containing inserts (average size: 1–2 kb). The library (≈700 000 cfu) was plated onto LB plates containing ampicillin and chloramphenicol and probed with a ~280 bp PCR fragment (roughly encompassing the TM-2 to TM-4 region of GPCRs) which has been shown to hybridize to a broad set of GPCRs under reduced stringency conditions. Hybridization was conducted at 32°C for 18 h in 5×SSPE buffer containing 50% formamide, 4×Denhardt's solution, 0.1% SDS, 10% dextran sulfate, 30 μg/ml sheared salmon-sperm DNA with 2×10^6 cpm/ml of ^{32}P-labeled probe. The probe was radiolabeled by random-priming with [α-^{32}P]dCTP to a specific activity of greater than 10^9 dpm/μg. The filters were then washed in 1×SSC, 0.1% SDS at 55°C and exposed to film (Kodak X-omat) for 48 h. Two independent positive clones were identified (designated 27A and 16.6) and subjected to further analysis. DNA was prepared from overnight cultures using the Wizard DNA Purification System (Promega Corp., Madison, WI) and subjected to automated sequence analysis using the PRISM Dye Deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 377 instrument. Initial sequencing primers were complementary to the T7 and SP6 promoter sites in pcDNA-3, additional primers were made complementary to the insert DNA. Database searches (Genbank, EMBL, Swiss-Prot, PIR, dEST, Prosite, dbGPCR), sequence alignments, and analysis of the GAL receptor nucleotide and protein sequences were carried out using the GCG Sequence Analysis Software Package (Madison, WI; PileUp, peptide structure and motif programs), FASTA and BLAST search

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programs, and the PC/Gene software suite from Intelligenetics (San Francisco, CA; protein analysis programs).

2.2. Expression of galanin receptor in COS-7 cells

The *Eco*RI inserts from clones 27A and 16 were subcloned into the mammalian expression vector pCI.neo (Promega Biotech, Madison WI). Clones in the correct orientation were selected following a digest with *Bam*HI. DNA was prepared using the Qiagen Maxi protocol (Qiagen, Chatsworth, CA) and transfected into COS-7 cells by electroporation. Briefly, 0.85 ml of COS-7 cells in Ringer's buffer (1.2×10^7/ml) and 20 μg of DNA were mixed in a 0.4 mm electroporation cuvette (BioRad, Hercules, CA) and current (960 μF, 260 V) was applied using a BioRad Electroporator device. Cells were transferred to a T-180 flask (Corning) with fresh media and expression was allowed to proceed for 72 h.

2.3. Membrane preparation and radioligand binding assay

Membranes were prepared from transfected cells following disruption in enzyme-free dissociation solution (Specialty Media, Lavallette, NJ) in a Dounce homogenizer in ice-cold membrane buffer (10 mM Tris-HCl, pH 7.4, 10 mM PMSF, 10 μM phosphoramidon, and 40 μg/ml bacitracin). After a low-speed ($1100\times g$, 10 min at 4°C) and a high-speed centrifugation ($38\,700\times g$ for 15 min at 4°C), membranes were suspended in buffer and the protein concentration determined (Bio-Rad assay kit). Binding of ^{125}I-labeled human galanin (sp. act.=2200 Ci/mmol, DuPont NEN) was measured in membranes using a buffer of 25 mM Tris-HCl, pH 7.4, 0.5% BSA, 2 mM $MgCl_2$, 40 μg/ml bacitracin, 4 μg/ml phosphoramidon, and 10 μM leupeptin in a total volume of 0.25 ml. 70 pM ^{125}I-labeled human galanin was used. Reactions were initiated by the addition of membranes and the incubation was allowed to proceed at room temperature for 1 h. Non-specific binding was defined as the amount of membrane-bound radioactivity remaining in the presence of 1 μM cold GAL. In competition studies various concentrations of peptides (hGal, pGal, hGal(1–16), rGAL(2–29), rGAL(3–29), hGal(1–19) or chimeric peptides (C7, M15, M40, M35) were included along with ^{125}I-labeled hGal (70 pmol). Incubations were terminated by rapid filtration through GF/C filters which had been presoaked with 0.1% polyethylamine using a TOMTEC (Orange, CT) cell harvester. The results were analyzed using the Prism software package (GraphPad, San Diego, CA).

2.4. Northern and Southern blot analysis

Poly(A)$^+$ mRNA was isolated from various rat tissues and cell lines as described above. Five micrograms of poly(A$^+$) RNA from each tissue was denatured and run on a formaldehyde–MOPS gel and transferred directly after electrophoresis to a ZetaProbe GT membrane (BioRad) by capillary transfer in 20×SSC. Northern blots were prehybridized at 42°C for 2 h in 5×SSPE, 2×Denhardt's solution, 100 μg/ml salmon sperm DNA, 0.1% SDS, and 50% formamide. Hybridizations were carried out at 42°C for 20 h in the above solution containing 10% dextran sulfate and 1×10^6 cpm/ml of radiolabeled probe, random-prime labeled with [α-^{32}P]dCTP to a specific activity of greater than 10^9 dpm/μg. The probe used was a PCR fragment of ~600 bp encompassing the majority of the ORF predicted by clone 27A (nt 1–618 of the ORF) beginning at the initiation codon and ending at transmembrane domain 5. The Northern blots were washed in 2×SSC, 0.1% SDS at room temperature for 2×15 min, in 1×SSC, 0.1% SDS at 65°C for 2×15 min, and 0.1 xSSC, 0.1% SDS at 65°C for 1 h. RNA size standards were in vitro transcribed RNA markers (BRL). Nylon membranes containing *Eco*RI-digested genomic DNA from several species (Clontech; 10 μg/lane) were hybridized for 24 h at 30°C in 6×SSPE, 10×Denhardt's, 1% SDS, and 50% formamide using a PCR-generated fragment of ~330 bp from the third intracellular loop to the C-terminal intracellular domain (nt 667–999). Southern blots were washed twice with room temperature 6×SSPE for 15 min each, twice with 55°C 6×SSPE for 15 min each, and twice with 55°C 4×SSPE for 30 min each.

3. Results and discussion

To isolate GAL receptor family members that may mediate the effects of GAL on the CNS, a rat hypothalamic cDNA library was screened under moderate hybridization conditions. The radiolabeled probe utilized has been shown to hybridize to several GPCRs under reduced stringency conditions. In addition to the GAL receptor described in this report, full-length clones encoding neurotensin receptor type 2 and the growth hormone secretagogue receptor type 1a have also been isolated with this probe (unpublished observations). Out of ~750 000 independent clones screened, two clones, termed 27A and 16.6, were identified as positive through repeated rounds of screening. As outlined in Fig. 1A, clone 27A contains the complete ORF for a 7-TM receptor divided into two exons by an unspliced intron of ~500 bp while clone 16.6 contains a virtually identical ORF as a single contiguous reading frame. A perfectly conserved splice donor site (G/gt) is found at nucleotide 368 which coincides with the second residue of the GPCR signature aromatic triplet RY (Fig. 1D,E). Exon 1 of clone 27A encodes the N-terminal extracellular domain through predicted TM-3, while exon 2 encodes the second predicted extracellular loop through the C-terminal intracellular domain. Both clones encode a polypeptide of 372 amino acids (predicted molecular mass of 40.6 kDa) which differ by a single amino acid (residue 37 is L in clone 27A, F in clone 16.6) in the predicted TM-1, suggesting a possible polymorphism. Searches of nucleic acid and protein databases revealed that the nucleotide sequence is unique and most closely related to the rat GALR1 receptor with 55% nucleic acid and 38% protein sequence identity.

An alignment of the protein sequences for rat GALR1 and GALR2 is given in Fig. 2. Several conserved features of GPCR were also identified in the rat GALR2: the signature aromatic triplet sequence (Glu–Arg–Tyr) adjacent to TM-3, Cys-98 and Cys-153 in the first two extracellular loops capable of disulfide bonding, putative amino-terminal N-glycosylation sites (Asn–Xaa–Ser/Thr), phosphorylation sites in the carboxyl-terminus and the third cytoplasmic loop, and conserved proline residues in TM-4, -5, -6 and -7. Compared to rat GALR1, rat GALR2 is overall 26 amino acids longer, the difference in length being centered at the amino terminus (GALR2 is eight amino acids shorter than GALR1), and the carboxyl terminal tail (GALR2 is longer by 34 amino acids).

Membranes were prepared from COS-7 cells transiently transfected with GALR2, using either clone 27A or 16.6 (both gave similar results) in order to assay ^{125}I-labeled human GAL binding. As shown in Fig. 3A, saturable and specific high-affinity binding was observed to a single class of non-interacting sites with a $K_D=0.23\pm0.05$ nM and B_{max} of 2.38 ± 0.11 pmol/mg membrane protein. No binding was observed in mock (vector only) transfected cells. To compare the pharmacological profiles of rat GALR1 and GALR2, competition studies were performed with GAL peptides (different

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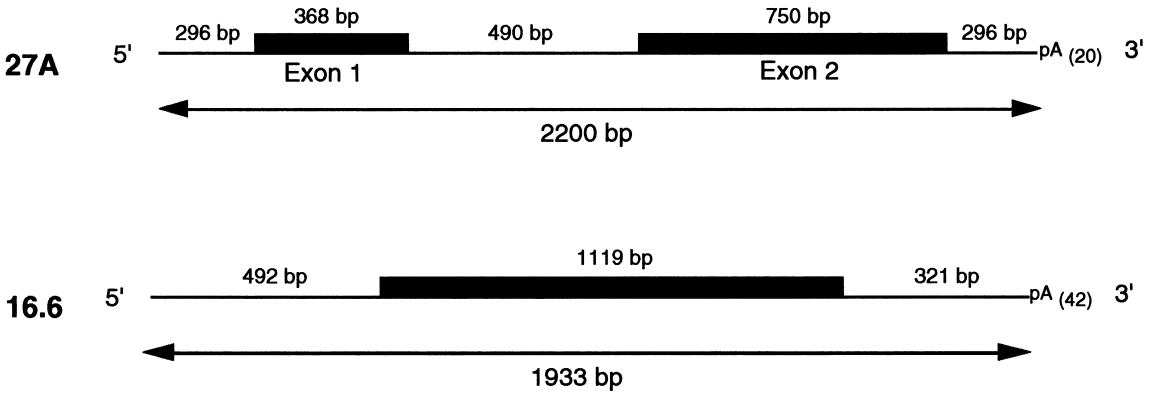
Fig. 1. A: Schematic representation of rat GALR2 clones 27A and 16.6. Clone 27A is presumably a precursor mRNA containing an unspliced intron dividing the ORF into two exons. Clone 16.6 encodes a contiguous ORF for the complete GALR2 polypeptide. The putative splice junctions in clone 27A (nt 368) are perfectly conserved in clone 16.6 which generates an AGG codon (Arg-123). B: Nucleotide and deduced amino acid sequence of rat GALR2 (clone 27A). Predicted transmembrane regions are underlined. Sites of potential N-linked glycoysylation are denoted by * whereas phosphorylation sites are given by $^+$.

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      *                                 *  +
  1 ATG AAT GGC TCC GGC AGC CAG GGC GCG GAG AAC ACG AGC CAG GAA GGC GGT AGC GGC GGC TGG CAG CCT GAG GCG  75
  1 M   N   G   S   G   S   Q   G   A   E   N   T   S   Q   E   G   G   S   G   G   W   Q   P   E   A   25

 76 GTC CTT GTA CCC CTA TTT TTC GCG CTC ATC TTC CTC GTG GGC ACC GTG GGC AAC GCG CTG GTG CTG GCG GTG CTG  150
 26 V   L   V   P   L   F   F   A   L   I   F   L   V   G   T   V   G   N   A   L   V   L   A   V   L   50

151 CTG CGC GGC GGC CAG GCG GTC AGC ACC ACC AAC CTG TTC ATC CTC AAC CTG GGC GTG GCC GAC CTG TGT TTC ATC  225
 51 L   R   G   G   Q   A   V   S   T   T   N   L   F   I   L   N   L   G   V   A   D   L   C   F   I   75
                                             +
226 CTG TGC TGC GTG CCT TTC CAG GCC ACC ATC TAC ACC CTG GAC GAC TGG GTG TTC GGC TCG CTG CTC TGC AAG GCT  300
 76 L   C   C   V   P   F   Q   A   T   I   Y   T   L   D   D   W   V   F   G   S   L   L   C   K   A   100

301 GTT CAT TTC CTC ATC TTT CTC ACT ATG CAC GCC AGC AGC TTC ACG CTG GCC GCC GTC TCC CTG GAC AG          368
101 V   H   F   L   I   F   L   T   M   H   A   S   S   F   T   L   A   A   V   S   L   D   R          123

  369   gtaaaggacccagaaagaaacatccagtatgcccggagggatcttgactggaaaagactgaatcctggtctggtgaccttt
  449   agttccctgccctttcacatcacttggacattcccacagaagagcggtgaagaggcggtggtccttattctcctctggtt
  529   tccactgagtgcaacatgtgcgtcctgagtacgctggagggactcacaaaatttcagctttctttaggagtttccttgct
  609   gtagtttgacccaagtcttctccaggtttctgtcagaacctcaggcatgagggatctgcctcccctggttgtcaccagag
  689   gataacaatcactgccccccagaaatccagacagattctacaacttttagtcttcggtgttttgggggtgccccttcacgt
  769   ggagtaggtcggtggccacattcccaggagtgacaatagcctagcagtgaatcctctcgcttagctgatgccccccact
  849   gtccccacag
                                          +
859   G TAT CTG GCC ATC CGC TAC CCG CTG CAC TCC CGA GAG TTG CGC ACA CCT CGA AAC GCG CTG GCC GCC ATC GGG  931
124     Y   L   A   I   R   Y   P   L   H   S   R   E   L   R   T   P   R   N   A   L   A   A   I   G  147
                                                                                        *
932 CTC ATC TGG GGG CTA GCA CTG CTC TTC TCC GGG CCC TAC CTG AGC TAC TAC CGT CAG TCG CAG CTG GCC AAC CTG 1006
148 L   I   W   G   L   A   L   L   F   S   G   P   Y   L   S   Y   Y   R   Q   S   Q   L   A   N   L  172

1007 ACA GTA TGC CAC CCA GCA TGG AGC GCA CCT CGA CGT CGA GCC ATG GAC CTC TGC ACC TTC GTC TTT AGC TAC CTG 1081
173 T   V   C   H   P   A   W   S   A   P   R   R   R   A   M   D   L   C   T   F   V   F   S   Y   L  197
                                          +
1082 CTG CCA GTG CTA GTC CTC AGT CTG ACC TAT GCG CGT ACC CTG CGC TAC CTC TGG CGC ACA GTC GAC CCG GTG ACT 1156
198 L   P   V   L   V   L   S   L   T   Y   A   R   T   L   R   Y   L   W   R   T   V   D   P   V   T  222
              +                           +
1157 GCA GGC TCA GGT TCC CAG CGC GCC AAA CGC AAG GTG ACA CGG ATG ATC ATC ATC GTG GCG GTG CTT TTC TGC CTC 1231
223 A   G   S   G   S   Q   R   A   K   R   K   V   T   R   M   I   I   I   V   A   V   L   F   C   L  247

1232 TGT TGG ATG CCC CAC CAC GCG CTT ATC CTC TGC GTG TGG TTT GGT CGC TTC CCG CTC ACG CGT GCC ACT TAC GCG 1306
248 C   W   M   P   H   H   A   L   I   L   C   V   W   F   G   R   F   P   L   T   R   A   T   Y   A  272

1307 TTG CGC ATC CTT TCA CAC CTA GTT TCC TAT GCC AAC TCC TGT GTC AAC CCC ATC GTT TAC GCT CTG GTC TCC AAG 1381
273 L   R   I   L   S   H   L   V   S   Y   A   N   S   C   V   N   P   I   V   Y   A   L   V   S   K  297
                                                                                    +
1382 CAT TTC CGT AAA GGT TTC CGC AAA ATC TGC GCG GGC CTG CTG CGC CCT GCC CCG AGG CGA GCT TCG GGC CGA GTG 1456
298 H   F   R   K   G   F   R   K   I   C   A   G   L   L   R   P   A   P   R   R   A   S   G   R   V  322
                      *               +
1457 AGC ATC CTG GCG CCT GGG AAC CAT AGT GGC AGC ATG CTG GAA CAG GAA TCC ACA GAC CTG ACA CAG GTG AGC GAG 1531
323 S   I   L   A   P   G   N   H   S   G   S   M   L   E   Q   E   S   T   D   L   T   Q   V   S   E  347
                                      *               +
1532 GCA GCC GGG CCC CTT GTC CCA CCA CCC GCA CTT CCC AAC TGC ACA GCC TCG AGT AGA ACC CTG GAT CCG GCT TGT 1606
348 A   A   G   P   L   V   P   P   P   A   L   P   N   C   T   A   S   S   R   T   L   D   P   A   C  372

1607 TAA                                                                                              1609
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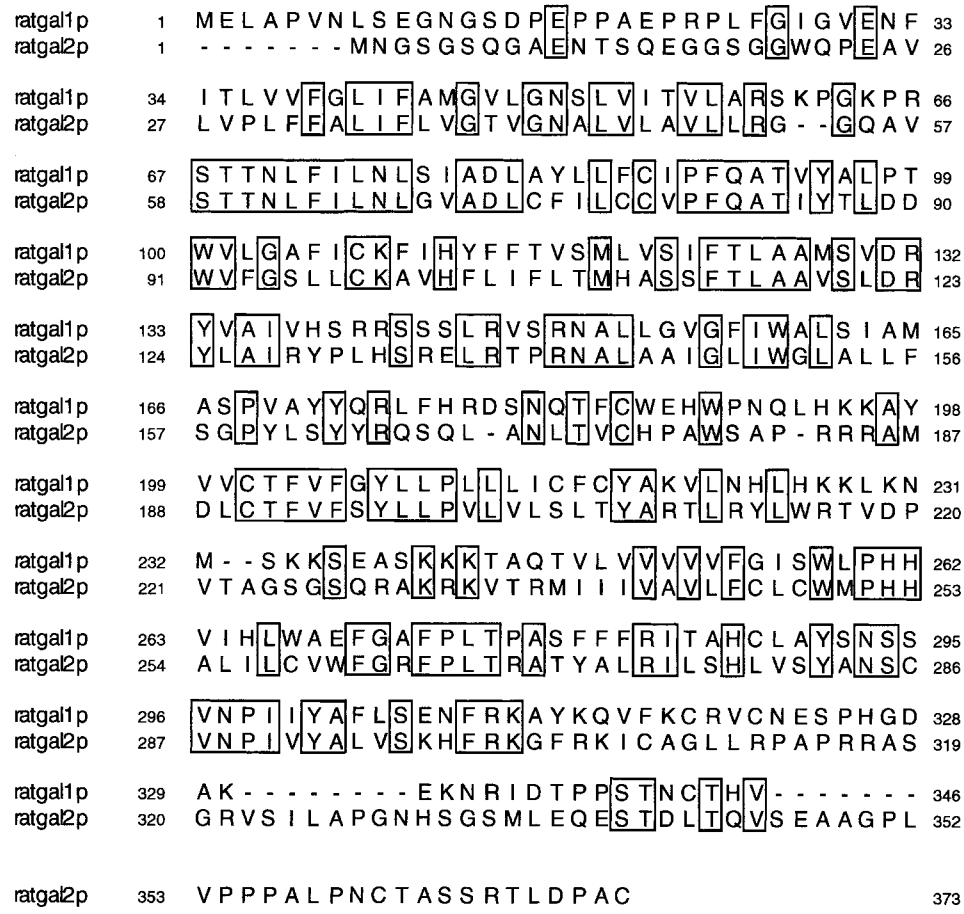

Fig. 2. Alignment of amino acid sequences for rat GALR1 and GALR2 (clone 27A). The PileUp program (GCG Program Suite, Madison, WI) was utilized to compare sequences.

species or truncated peptides) and the chimeric peptides. As given in Table 1, the rank order of potency for the tested chimeric peptides is similar between the two receptor types: M35 > M40 > C7, galantide. However, GALR2 shows a 10-fold lower affinity for ^{125}I-labeled human GAL, whether assaying rat, human or porcine GAL (rat GALR1 has an affinity for human GAL of 0.02 nM; unpublished observations). Both GALR1 and GALR2 share a requirement for the amino terminal residues of GAL for high-affinity binding as evidenced by the lack of binding to rGAL(3–29). Thus both of these receptors are distinct from the receptors proposed to exist in the pituitary and in the gut which are insensitive to delections of the first two amino acids of GAL. There is a subtle difference in the affinity of GALR2 for porcine GAL, while GALR1 does not distinguish between the human, rat and porcine GALs. This suggests that the GALR2 receptor may respond in a different way to changes in the carboxy terminal region of native GAL. Differences in IC_{50} values were noted for most peptides against GALR1 and GALR2 such that between 5- to 30-fold higher concentrations of test peptide was needed for 50% inhibition against GALR2. One explanation for the overall lower affinity of ^{125}I-labeled human GAL to rat GALR2 may be species differences: rat GALR2 shows a much higher affinity for porcine GAL versus human GAL.

Table 1
Pharmacology of rat GALR1 and GALR2

	IC_{50} (nM)	
	Rat GALR1	Rat GALR2 (clone 27A)
Porcine GAL	0.06	0.46
Human GAL	0.07 ± 0.01	1.3 ± 0.5
Rat GAL(2–29)	7.2	2.9 ± 1.3
Rat GAL(3–29)	> 1000	> 1000
Porcine GAL(1–16)	0.27 ± 0.18	3.0
Galantide (M15)	1.0 ± 1.1	28 ± 3.5
C7	4.9 ± 3	23 ± 13
M40	0.9 ± 0.6	1.9 ± 0.14
M35	0.01	0.43 ± 0.18

IC_{50} values were calculated from displacement curves of ^{125}I-labeled human GAL binding using non-linear regression analysis (Prism V2.0, GraphPad Software, San Diego, CA).

Transcripts encoding GALR2 were readily detected in several rat tissues, with expression in RINm5F cells and uterus being most prominent (Fig. 4A). Expression of GALR2 in RINm5F cells suggests that the previously studied pharmacological properties of these cells was complicated by a mixed receptor population composed of at least two receptor types. Genomic Southern analysis suggests that GALR2 is encoded by a single conserved gene, as a simple hybridization pattern, indicative of a single gene only was noted in several species (see legend to Fig. 4 for details).

In summary, we have isolated a full-length cDNA encoding a second member of the GAL receptor family. Despite the amino acid differences between the GALR1 and GALR2 re-

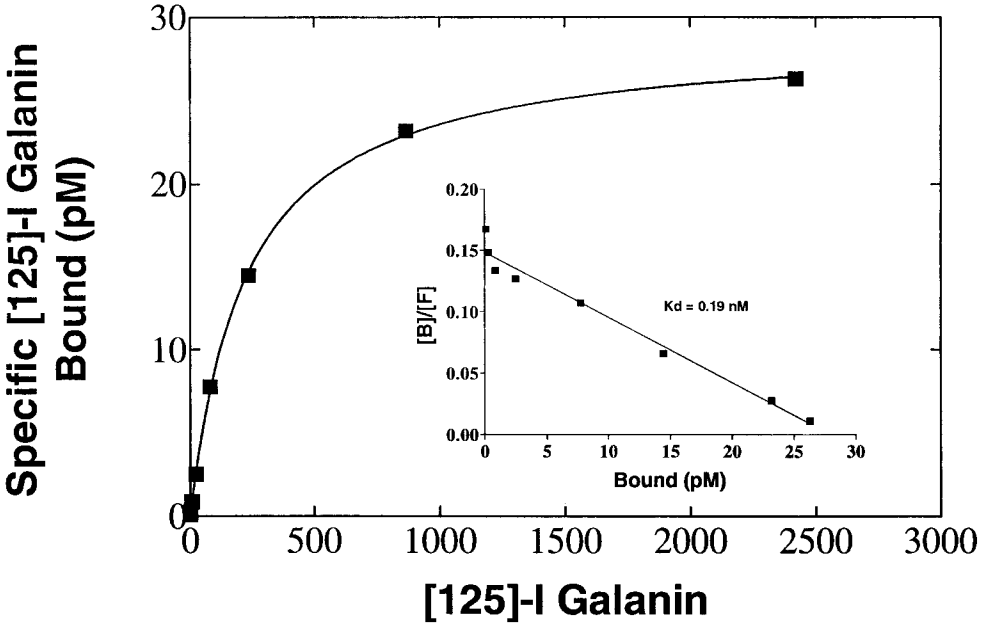

Fig. 3. Pharmacological characterization of rat GALR2. Clone 27A was sub-cloned into a mammalian expression vector, transfected into COS-7 cells, and 72 h following tranfection, cell membranes were assayed for human ^{125}I-labeled GAL binding. For saturation isotherm and Scatchard analysis, 3 μg of membrane protein was used for each assay point (triplicate measurements).

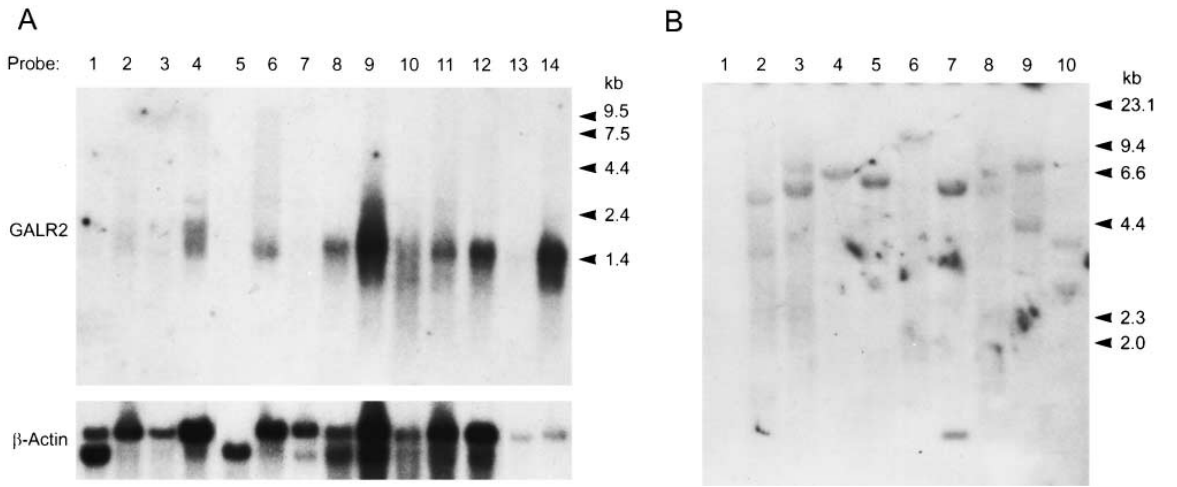

Fig. 4. Northern and Southern blot analysis of rat GALR2 expression. A: An RNA blot was prepared using poly(A$^+$) mRNA (1 μg/lane) from: lane 1, heart; lane 2, kidney; lane 3, liver; lane 4, lung; lane 5, skeletal muscle; lane 6, prostate; lane 7, testes; lane 8, vas deferens; lane 9, uterus; lane 10, ovary; lane 11, stomach; lane 12, large intestine; lane 13, pancreas; lane 14, RIN m5F cells. Upper panel: the radiolabeled probe utilized encompasses most of the GALR2 ORF from the initiation codon through TM-5. Following high-stringency post-hybridizational washing, the blot was exposed to X-ray film for 3 days at −70°C. RNA size marker (Life Technologies) are 9.5, 7.5, 4.4, 2.4, and 1.35 kb. Lower panel: the same blot hybridized with a radiolabeled actin probe to normalize mRNA loaded. B: A commercial genomic Southern blot (*Eco*RI-digested DNA, 10 μg/lane) was hybridized with a 3′-fragment of the rat GALR2 ORF (third intracellular loop to C-terminal intracellular domain). Post-hybridizational washing stringencies were at 55°C 4×SSPE after which the filters were dried and exposed to X-ray film for 5 days at −70°C. Lane 1, lambda *Hin*dIII markers (23.1, 9.4, 6.6, 4.4, 2.3, 2.1 kb); lane 2, human; lane 3, monkey; lane 4, rat; lane 5, mouse; lane 6, dog; lane 7, cow; lane 8, rabbit; lane 9, chicken; lane 10, yeast.

ceptors their pharmacological profile is remarkably similar. Therefore in order to distinguish what role each receptor plays in mediating the biological effects of GAL, one must await the development of GAL receptor subtype-specific antagonists or targeted disruption of each receptor gene.

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