ACCELERATED COMMUNICATION

Molecular Cloning and Pharmacological Characterization of a New Galanin Receptor Subtype

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SUMMARY

Galanin, a 29–30-amino acid neuropeptide, is widely distributed in central and peripheral systems and mediates a variety of physiological functions. Pharmacological studies have suggested the existence of multiple receptor subtypes but only the type I (GalR1) galanin receptor has been cloned. Now we report the cloning by a combination of sib selection and rapid amplification of cDNA ends of a cDNA encoding a new galanin receptor (GalR2) from rat hypothalamus. The receptor is 372 amino acids in length and shares only 40% homology with the rat GalR1 receptor. It contains seven putative transmembrane domains with the amino and carboxyl termini being least identical to GalR1. Northern blot analyses revealed a 2-kilobase pair mRNA species distributed in several tissues, suggesting a broader functional spectrum than GalR1. ^{125}I-Labeled human galanin binding to rat GalR2 receptor expressed in COS-1 cells was saturable (K_d = 0.59 nM) and could be displaced by galanin, several galanin fragments, and chimeric peptides. The pharmacological profiles of GalR1 and GalR2 receptors were distinguishable by galanin fragment(2–29), which bound the cloned GalR2 receptor with markedly higher affinity than the GalR1 receptor. Activation of the cloned receptor by galanin led to inhibition of forskolin-stimulated intracellular cAMP production. The cloning of this new receptor subtype should provide further insights into the mechanisms by which galanin mediates its diverse physiological functions. The identification of galanin(2–29) as a receptor-specific ligand should enhance the understanding of specificity of galanin-receptor interactions.

Galanin, a neuropeptide 29–30 amino acids in length, mediates diverse and crucial physiological functions (1, 2). Centrally it effects cognition, nociception, and feeding behavior (3–5), and peripherally, it mediates regulations of insulin release and muscle contraction (6, 7). Galanin exerts its function through binding to its receptors. Biochemically, a galanin receptor has been characterized as a glycoprotein with an apparent molecular mass of 54 kDa (8) and a signaling pathway linked through G proteins to the inhibition of adenylyl cyclase through a pertussis-toxin sensitive mechanism (9).

Pharmacological studies with several peptidic agonists and antagonists of galanin receptor suggest the existence of more than one receptor subtypes (10–12). The galanin GalR1 receptor has been cloned from human melanoma cells (13), rat Rin14B insulinoma cells (14), and rat brain (15). The receptor belongs to the G protein-coupled receptor superfamily, characterized by seven hydrophobic transmembrane domains

(16), and displays inhibitory effects on forskolin-stimulated intracellular cAMP production in cells either stably or transiently expressing GalR1 receptor. In this report, we describe the cloning of a novel galanin receptor subtype by a combination of sib selection of a rat hypothalamus cDNA library and RACE. The receptor has been expressed in COS-1 cells and displays a distinct pharmacological profile that is distinguished from that of GalR1 by its high affinity for the fragment galanin(2–29).

Materials and Methods

ABBREVIATIONS: GalR, galanin receptor; RACE, rapid amplification of cDNA ends; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; GMAP, galanin messenger-associated peptide(1–41); PCR, polymerase chain reaction; bp, base pair; kb, kilobase pair; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

acctgtccagggagacgg; oligo124, 5′-gcactggcagcaggtagctaaagac; oligo129, 5′-tgctagtcctcagtctgacctat; oligo139, 5′- ggagcggcgtccactttggtgatac; and oligo137, 5′-acgttcaagcataaccccccagg. Rat multiple tissue Northern and rat genomic DNA blots were obtained from Clontech (Palo Alto, CA). Rat galanin, rat galanin (1–16), M40, C7, (D-Thr[6],D-Trp[8,9])galanin(1–15)ol, and galantide (M15) were purchased from Peninsula Laboratories (Belmont, CA). Galanin(2–29) was custom synthesized by Biosynthesis, Inc.). Galantide, galanin(1–13)-bradykinin(2–9) (M35), and galanin messenger-associated peptide(1–41) were from Sigma.

PCR sib selection. PCR and galanin receptor-specific primers were used to screen pools of a rat hypothalamus cDNA library. When positive pools were identified they were subdivided and screened similarly by the PCR screening until the final round of screening in which individual colonies were selected by analysis of plasmid minipreps. The rat cDNA library was constructed as follows. Total RNA from rat hypothalamus was extracted with Tri-reagent-RNA/DNA/protein Isolation Reagent (Molecular Research Center, Cincinnati, OH). Poly(A)$^+$ RNA from the total RNA was purified with an mRNA purification kit that employs oligo(dT)-cellulose chromatography (Pharmacia, Piscataway, NJ). Double strand cDNA was synthesized from the poly(A)$^+$ RNA with a Marathon cDNA amplification kit (Clontech). A portion of the cDNA was blunt-end ligated with adaptor containing a *Bst*XI restriction site. The *Bst*XI adaptor-linked cDNA was then ligated into pCDNA3 vector predigested with *Bst*XI.

PCR and RACE amplification of cDNA fragment. Unless otherwise specified, PCR was always run with KlenTaq polymerase that possesses proof reading activity (Clontech) and a cycling profile of 94° for 1 min, 65° for 1 min, and 72° for 2 min (40 cycles). For RACE, nested oligonucleotides specific to the rat GalR2 cDNA and nested adaptor primers were used. Approximately 0.1 μg of cDNA from tissues was used as template in PCR and RACE, and 1 μl of overnight *Escherichia coli* cell culture was used in PCR sib selection.

DNA sequencing and analysis. DNA sequencing was performed with ABI Prism dye termination DNA sequencing reagents and an ABI automated sequencing apparatus (Perkin Elmer, Branchburg, NJ). DNA and protein sequence comparisons were performed with the DNA* software (DNAstar, Madison, WI).

Transfection of COS-1 cells. COS-1 cells grown in DMEM supplemented with 10% fetal calf serum (FCS) in were split 1:6 into 150-mm dishes (Nunc) 3 days before transfection. On the day of transfection, the cells were approximately 90% confluent and trypsinized off plates and washed two times with PBS without Mg^{2+} and Ca^{2+}. The cells were resuspended in Krebs- Ringer's buffer at a density of approximately 1.2 × 10^7 cells/ml. Twenty micrograms of plasmid DNA were diluted in Krebs-Ringer's buffer (100 μl final volume), mixed with 0.7 ml of the COS-1 cells in a 0.4-cm electroporation cuvette (Bio-Rad, Hercules, CA), then chilled on ice for 5 min. The cells were electroporated at 960 μF × 260 V (time constant approximately 18) followed by incubation on ice for 10 min. The cells were incubated in DMEM with 10% fetal calf serum in a 150-mm plate.

Receptor membrane preparation. Three days after the transfection of COS-1 cells, the cell medium was removed, and the cells were washed three times with PBS (no Ca^{2+}/Mg^{2+}). To each plate 5 ml of 5 mM HEPES buffer, pH 7.4, 0.1 mM PMSF, and 0.1 mg/ml bacitracin were added and incubated at room temperature for 15 min. The cells were scraped off plates and centrifuged at 13,000 × g for 15 min at 4°. The cell pellet was resuspended in 2 ml of 25 mM Tris-Cl, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride by vortexing and dispersed with a syringe attached to a 23-gauge needle. Protein concentrations were determined by a BCA method (Pierce, Rockford, IL).

^{125}I-Galanin binding assay. Binding of ^{125}I-human-galanin to the membrane preparations was performed in a buffer containing 25 mM Tris-Cl (pH 7.4), 1% bovine serum albumin (radioimmunoassay grade), 0.1% bacitracin, 2 μg/ml leupeptin, 0.1 mM PMSF, and 10 mM MgCl$_2$. Ligand saturation plots were performed using 10 μg of the membrane protein in a total volume of 200 μl using 3 μM cold galanin

to determine nonspecific binding. Peptide competition studies were performed in a total volume of 200 μl, containing 10 μg of membrane protein and 0.3 nM ^{125}I-human galanin. Incubations were at room temperature for 1 hr and terminated by rapid vacuum filtration through Multiscreen FB Filter Plates (Millipore, Bedford, MA) that had been pretreated with 0.3% polyethylenimine. The filters were then washed three times with 100 μl of PBS (pH 7.4). All data were analyzed using nonlinear regression software (Prism, GraphPad, San Diego, CA) and the K_i calculated according to the method of Cheng and Prusoff (17).

Cyclic AMP analysis. COS-1 cells grown in 150-mm diameter plates were transfected as described above. After a 3-day post-transfection growth, the cells were washed with PBS, scraped off the plates, and resuspended at a density of 2×10^5 cells/100 μl of buffer containing 75 mM Tris-Cl, pH 7.4, 250 mM sucrose, 12.5 mM MgCl, 1.5 mM EDTA, and 0.2 mM sodium metabisulfite. Cells were incubated in DMEM for 1 hr at 22° in 130 μl of the buffer plus 0.1 mM forskolin and 0.2 mM 3-isobutyl-1-methylxanthine with rat galanin or peptides at the indicated concentrations. The cells were then lysed by boiling for 5 min and chilled on ice. The concentration of cAMP in the cell lysates was determined by scintillation proximity assay cAMP (Amersham, Arlington Heights, IL). The data were analyzed with the Prism nonlinear regression method to obtain maximum inhibition and EC$_{50}$ values.

Northern blot analysis of rat GalR2. Rat multiple tissue Northern blot was hybridized for 15 hr at 68° in an ExpressHyb solution (Clontech) using ^{32}P-labeled rat GalR2 cDNA (~420 bp, excised out from clone 9798sw8) as a probe [labeled with a random priming kit (Life Technologies); specific activity = 10^9 cpm/μg]. After hybridization, the blot was washed with wash solution I (2 × SSC, 0.05% sodium dodecyl sulfate) for 30 min at room temperature then with wash solution II (0.1 × SSC, 0.1% sodium dodecyl sulfate) for approximately 20 min at 45°. The blots were then wrapped with Saran Wrap and exposed to Kodak BioMax films for 1 week. The same blot was stripped and similarly hybridized with ^{32}P-labeled actin cDNA to ensure loading of poly(A)$^+$ mRNA from the tissues onto the blot.

Southern blot analysis. Blotted rat genomic DNA, loaded at 4 μg/lane (Rat Geno blot, no. 7611-1, Clontech), was hybridized with ^{32}P-labeled full-length rat GalR2 cDNA (random priming, specific activity = 5 × 10^9 cpm/μg DNA) at 60° overnight. The blot was washed in solution I for 30 min at room temperature and washed in solution II for 1.5 hr at 41° and another 40 min at 55°. A film was exposed to the blot for 4 days at −80°.

Results and Discussion

Galanin serves as a neurotransmitter and hormone that mediates diverse physiological functions in the central and peripheral systems. Pharmacological studies suggest that more than one receptor subtype exists, raising the possibility that multiple galanin receptors are involved in these various functions. To directly test this hypothesis, we used a molecular cloning approach to identify a non-GalR1 receptor from rat. Several PCR primers, either identical or degenerate, corresponding to the transmembrane regions of rat GalR1 cDNA, were used in PCR with rat lung cDNA as template, which was generated by reverse-transcription from rat lung poly(A)$^+$ mRNA. Rat lung was previously shown to contain no detectable GalR1 mRNA (14) thus was used to avoid preferred amplification of rat GalR1 cDNA. PCR with two primers, oligo97 and oligo98, yielded a band of approximately 420 bp. This band was cloned and found to contain rat GalR1 cDNA and various non-GalR1 sequences. One of the non-GalR1 clones (9798sw8) possessed highest homology to rat GalR1 cDNA at both nucleotide (65%) and amino acid levels

Fig. 1. Molecular cloning of a rat GalR2 cDNA. A, Isolation of the rat GalR2 cDNA. *Left,* five steps involved in the cloning; *long boxes,* clones obtained at each step by PCR, RACE, or by PCR-based sib selection. Pairs of primers used in the cloning steps are shown on the top (forward primer) and bottom (reverse primer) of each clone accompanied by their names. The lengths and relative positions of the clones are shown approximately to scale. ATG and *stop codon* indicate the initiation and termination sites for the translation of the receptor. A cycling profile of 92° for 0.5 min, 58° for 0.5 min, and 72° for 1 min (40 cycles) was used in step 1, and standard cycling was used in other PCRs (see Materials and Methods). B, Nucleic acid and amino acid sequences of the rat GalR2 receptor. Shown are the coding nucleotide sequence of the rat GalR2 cDNA, and underneath is the deduced amino acid sequence. *Right,* numbering of the nucleotide sequence; *underlined Roman letters,* putative transmembrane regions I–VII, assigned based on comparison with the rat GalR1 sequence (15) and hydrophobicity analysis (27). ∗, Potential *N*-linked glycosylation site; ●, two Cys residues that may form a disulfide bridge. C, Alignment of the amino acid sequence of rat GalR2 receptor with rat GalR1 receptor (15). The top sequence *pCR3.1-rGalR#20* shows the translated rat GAIR2 receptor encoded by clone pCR3.1-rGAIR#20, and the amino acid residues in rat GalR1 different from the GalR2 receptor are shown beneath. *Dotted lines,* amino acid residues identical to the GalR2 sequence. Gaps (-) are introduced to optimize the alignment. *Overlines,* the seven putative transmembrane domains.

(45%) (Fig. 1A). This clone was then considered as a portion of a candidate rat GalR2 cDNA.

Two primers specific to the rat GalR2 cDNA were designed (oligo112 and oligo113) and used in PCR to screen cDNA pools of a rat hypothalamus cDNA library (5000 clones/pool). After four rounds of PCR sib selection, a clone, C27#33, with comparable size and considerable sequence overlap with clone 9798sw8, was identified from pool C27 (Fig. 1A). The 3′ end of the clone was unique to rat GalR2 cDNA and allowed generation of another rat GalR2 specific primer (oligo124). Three rounds of PCR sib selection on the rat hypothalamus cDNA pools with primers oligo112 and oligo124 yielded a positive clone (B45-11-16) from pool B45 that had an insert size of 1.3-kb (Fig. 1A) and contained the sequence of clone C27#33. A search of the GenBank database revealed that an open reading frame in this clone possessed highest homology to rat GalR1 (≥90% of the length of GalR1 cDNA) but contained no stop codon (Fig. 1A). To obtain the 3′ end, RACE was performed by using a rat brain cDNA library linked with the RACE adaptor on the two ends as template (Clontech) and nested primers specific to rat GalR2 cDNA and the RACE adaptors. The final 3′ RACE product (Fig. 1A), obtained with primer oligo129 and the inner one of the two nested adaptor primers, overlapped the 3′ portion of clone B45-11-16 (Fig. 1A).

Full-length rat GalR2 cDNA was obtained by PCR with primers oligo139 and oligo137 and rat hypothalamus cDNA as a template. A strong single band PCR product (~1.2-kb, Fig. 1A) was obtained and ligated into a TA cloning vector, pCR3.1 (InVitrogen, San Diego, CA). The clone was named pCR3.1-rGalR#20. Fig. 1B shows the open reading frame and the deduced amino acid sequence for the encoded rat GalR2 receptor. The receptor is 372 amino acids in length, has a calculated molecular mass of 40.7 kDa, and contains seven putative transmembrane spanning regions typical of G protein-coupled receptors. Other features common to the G protein-coupled receptor superfamily are also present in the rat GalR2 amino acid sequence. There is a single potential N-linked glycosylation site in the amino-terminal region, two Cys residues in the first and second extracellular loops that form a putative disulfide bond in these receptors, and two Cys residues in the carboxyl-terminal region that may be involved in palmitoylation (Fig. 1B). The overlapping sequences between the independent clones B45-11-16 (cDNA), the 3′ RACE product in cloning step 4, and pCR3.1-rGalR#20 (Fig. 1A) are identical, indicating the absence of PCR artifacts in the coding sequence.

The amino acid sequence of the rat GalR2 receptor is significantly different from that of rat GalR1. The overall homology is 40% at the amino acid level as analyzed by the Jutun Hein method (18) (Fig. 1C). A search of the SwissProt databank showed similarity to rat cholecystokinin receptor (38%) (19), the human somatostatin receptor (37%) (20), and the human κ opioid receptor (31%) (21). Sequence alignment between rat GalR1 and rat GalR2 amino acid sequences shows the greatest similarity in the second, fifth, and seventh transmembrane regions, being 63, 52, and 52% identical, respectively. Least conserved regions appear at the amino- and the carboxyl-terminal regions, being 16% and 13% identical, respectively (Fig. 1C).

To study the distribution of the mRNA of the rat GalR2 receptor, Northern blot analysis was performed by hybridization of blotted poly(A)$^+$ RNA from several rat tissues (Fig. 2A) to ^{32}P-labeled 9798sw8 fragment (~420 bp). A single 2-kb mRNA transcript was detected in all the tissues with somewhat stronger signals in kidney, testis, skeletal muscle, and liver than in brain, heart, spleen, and lung. The strikingly wide distribution of rat GalR2 mRNA is in contrast to the distribution of rat GalR1 mRNA, which has been detected at significant levels only in brain and spinal cord. The data suggest that rat GalR2 receptor may play a broader physiological role than GalR1 receptor, by acting not only in the central nervous system but also in peripheral systems. Southern blot hybridization to rat genomic DNA digested with restriction enzymes with ^{32}P-labeled full-length rat GalR2 cDNA followed by high stringency washes shows a single band of 15, 4, and 2.1 kb with *Eco*RI, *Hin*dIII, and *Bgl*II, respectively (Fig. 2B). Two bands at 3.8 and 1.6 kb observed with *Bam*HI, and three bands at 2.7, 1, and 0.7 kb observed with *Pst*I, may have resulted from *Bam*HI and *Pst*I sites in the introns and exons in the coding region. The presence of one *Pst*I site identified in the cDNA sequence (Fig. 1B) is consistent with this explanation. Thus, GalR2 appears to exist as a single copy in the rat genome.
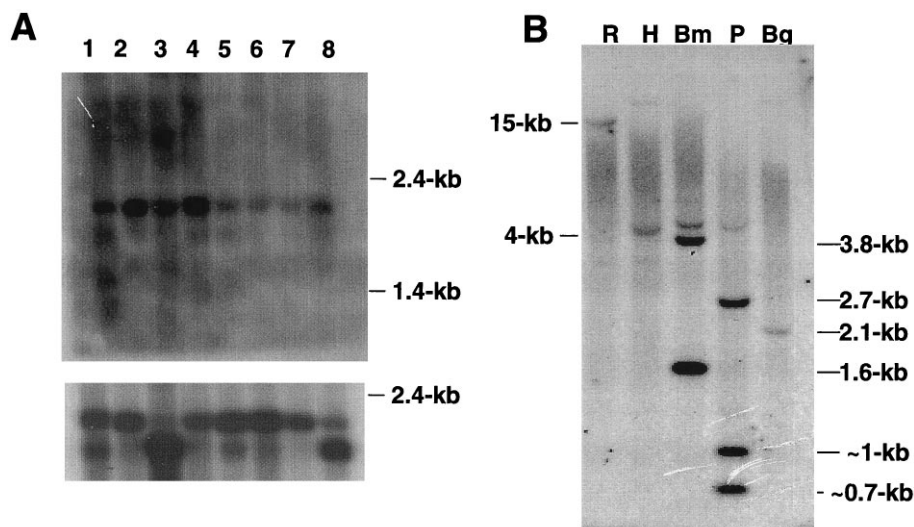
Fig. 2. A, Northern blot analysis. Northern blot containing rat poly(A)$^+$ mRNA isolated from several tissues was probed with a ^{32}P-labeled 420-bp fragment within the rat GalR2 cDNA coding region. *Lane 1*, testis; *lane 2*, kidney; *lane 3*, skeletal muscle; *lane 4*, liver; *lane 5*, lung; *lane 6*, spleen; *lane 7*, brain; and *lane 8*, heart. The blot was reprobed with ^{32}P-labeled actin cDNA to ensure comparable loading of mRNA from the tissues (*bottom*). B, Southern blot analysis. A Southern blot containing Sprague-Dawley rat kidney genomic DNA at 4 μg/lane was hybridized with full-length rat GalR2 cDNA (excised out of pCR3.1-rGalR#20 and gel-purified) labeled with ^{32}P. The lanes indicate the DNA digested with restriction enzymes: *R*, *Eco*RI; *H*, *Hin*dIII; *Bm*, *Bam*HI; *P*, *Pst*I; and *Bg*, *Bgl*II.

The ability of the expressed rat GalR2 receptor to bind radiolabeled galanin was examined by performing binding of ^{125}I-human galanin to membranes of COS-1 cells transfected with pCR3.1-rGalR#20 plasmid. The binding was saturable with high affinity, showing a K_d value of 0.59 ± 0.27 nM and a B_{max} value of 461 ± 153 fmol/mg of membrane protein (mean ± standard deviation; 4 independent transfections). A representative binding curve is illustrated in Fig. 3. Binding of the radioligand to cell membranes transfected with vector alone was negligible. Nonlinear regression analyses with one or two site-fit suggest that the ligand binds to a single class of binding sites in COS-1 cells transfected with the rat GalR2 cDNA. Similar results were obtained when the saturation binding assay was performed with ^{125}I-porcine galanin and rat GalR2 receptor prepared from COS-1 cells (K_d = 0.78 nM and a B_{max} = 595 fmol/mg of membrane protein).

To characterize the pharmacology of the rat GalR2 receptor, ^{125}I-human galanin binding to membranes prepared from COS-1 cells transfected with pCR3.1-rGalR#20 plasmid was competed with various galanin peptide analogs (Fig. 4). Rat galanin, galantide, galanin fragment(2–29), and galanin(1–13)-bradykinin(2–9) bound to rat GalR2 receptor at high affinity (Table 1 and Fig. 4). Lower affinity was found with galanin(1–13)-spantide (C7), galanin (1–16), and M40, with 16–32-fold lower affinities than native galanin. Two ligands, (D-Thr[6],D-Trp[8,9])galanin(1–15)ol and GMAP, whose affinities have not been reported for cloned galanin receptors, showed almost no binding to rat GalR2 receptor (Table 1). For comparison, a few ligands were tested with membranes of rat GalR1 receptor prepared from transiently transfected COS-1 cells (Table 1). Native galanin, galantide, and galanin(1–16) bound GalR1 with high affinity, whereas galanin(2–29) bound with lower affinity (by ~260-fold), consistent with the reported profile for the cloned GalR1 receptor (13–15). Cholecystokinin, somatostatin and dynorphin did not displace the radioligand in the assay.

Although most ligands displayed similar affinities between rat GalR1 and GalR2 receptors, we noted that the affinity of galanin(2–29) for GalR2 receptor was considerably higher than that for GalR1 receptor. Direct comparison of affinities
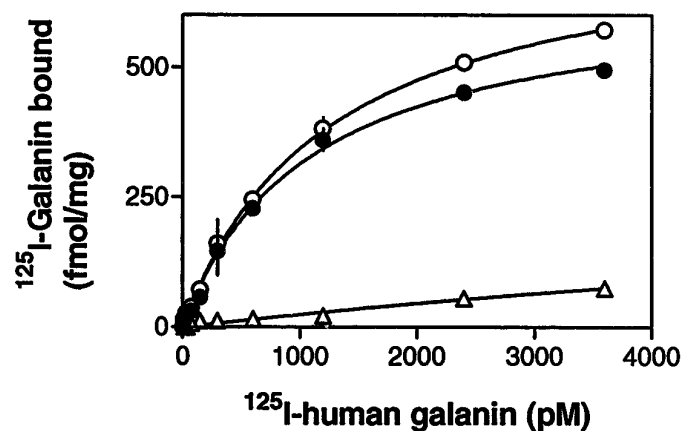

Fig. 3. Saturation analysis of ^{125}I-human galanin binding to membranes prepared from COS-1 cells transfected with clone pCR3.1-rGalR#20. *Points,* representative experiment performed in duplicate. *Specific binding curve,* fit with one-class binding sites on the receptor by nonlinear regression analysis. ○, total binding; △, nonspecific binding defined by the presence of 3 μM rat galanin; ●, rat GalR2 receptor-specific binding calculated as the difference between the total and nonspecific bindings.
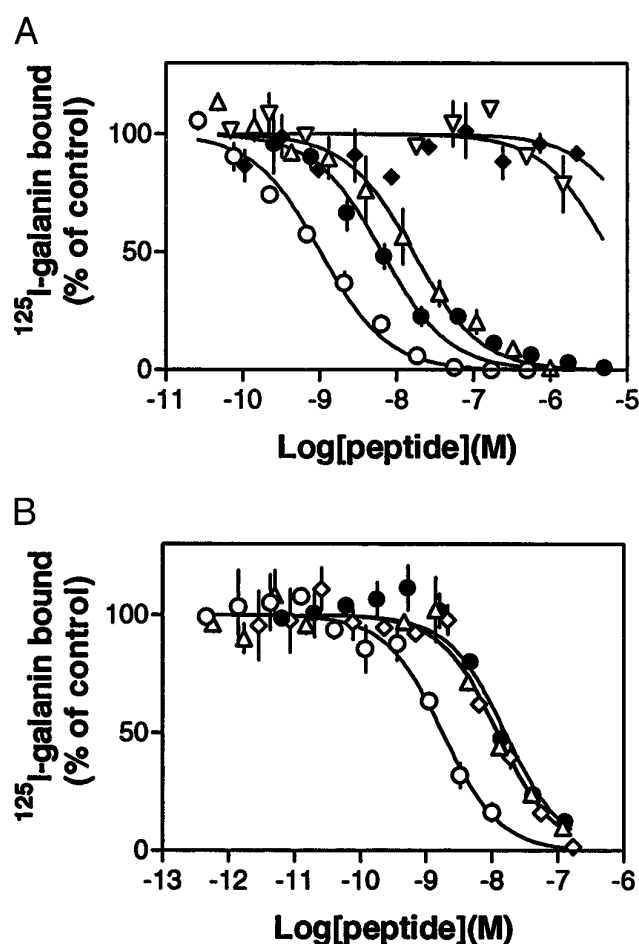

Fig. 4. Pharmacological profile of rat GalR2 in radioligand competition assays. ^{125}I-Labeled human galanin, at 0.3 nM, were displaced by indicated peptide ligands from rat GalR2 receptor-containing membranes of COS-1 cells transfected with clone pCR3.1-rGalR#20. Data shown are one representative experiment assayed in duplicate and reported as percent of binding in the absence of unlabeled peptide. A, Competition of ^{125}I-human galanin for GalR2 receptor with native galanin and galanin fragments. ○, Rat galanin; △, galanin(1–16); ●, galanin(2–29); ▽, (D-Thr[6],D-Trp[8,9])galanin(1–15)ol; ◆, GMAP. B, Same competition assays with galanin related chimeric peptides: ○, galantide; △, galanin(1–13)-bradykinin(2–9); ●, M40; and ◇, C7.

of galanin(2–29) for GalR1 and GalR2 revealed that the affinity for GalR1 was 42-fold lower than that for GalR2 (Table 1, Fig. 5). As controls in these experiments, galanin bound the two receptors with similar affinities (Fig. 5). The low affinity of galanin(2–29) for GalR1 indicates a stringent requirement for the amino-terminal Gly residue on the ligand for binding the receptor whereas this requirement is significantly lower for GalR2 receptor. Along with Trp2, Asp5, and Tyr9, the amino-terminal Gly of galanin has been identified as a key component of the pharmacocore for galanin binding to the GalR1 receptor (22). A hydrogen bond between the free NH_2 group of galanin and galanin receptor has been proposed, and acetylation of the NH_2 group resulted in reduction of the affinity of galanin by 20–60-fold (22). Mutagenesis of Glu271 of the human GalR1 receptor (Glu269 of rat GalR1) suggests this residue as the potential acceptor of the hydrogen bond from the amino terminus of galanin (23). The high binding affinity of galanin(2–29) to GalR2 receptor suggests that amino-terminal glycine is not important for the interac-

TABLE 1

Pharmacological profile of the cloned rat GalR2 receptor

K_i values were calculated from EC_{50} values ($K_i = EC_{50}/(1 + [^{125}I\text{-ligand}]/K_d)$) determined by nonlinear regression analysis of data from competitive displacement of ^{125}I-human galanin binding to membranes from COS-1 cells transiently transfected with clone pCR3.1-rGalR#20 for rat GalR2 receptor or pCDNA3-rGalR1 for rat GalR1 receptor. Data are presented as mean ± standard deviation of K_i from two to four independent experiments (numbers in parentheses) performed in duplicate. K_i(ligand)/K_i(galanin) denotes relative affinity of ligands in respect to that of native galanin. Rat GalR1 cDNA was obtained by RT-PCR from rat hypothalamus RNA and cloned into expression vector pDNA3. The cDNA was identical to the previously published sequence (14). The expression levels for rat GalR1 used in the assays were similar to those of rat GalR2 receptor expressed in COS-1 cells.

| Ligand	K_i	K_i (ligand)/ K_i (galanin)
	nM	
Rat GalR2 receptor		
Rat galanin(1–29)	0.56 ± 0.04 (3)	1.0
Galantide	1.17 ± 0.43 (4)	2.1
Rat galanin(2–29)	3.5 ± 0.5 (2)	6.3
Galanin(1–13)-bradykinin(2–9)	4.0 ± 3.0 (2)	7.1
Galanin(1–13)-spantide I, (C7)	9.1 ± 1.7 (2)	16.3
Rat galanin(1–16)	13 ± 12 (3)	23.2
M40	18 ± 18 (3)	32.1
(D-Thr[6], D-Trp[8,9])galanin(1–5)ol	>1250 (4)	>2200
GMAP (1–41)	>840 (2)	>1500
Rat GalR1 receptor		
Rat galanin(1–29)	0.33 ± 0.19 (2)	1.0
Galantide	0.85 ± 0.55 (2)	2.6
Rat galanin(1–16)	4.9 ± 2.3 (2)	14.8
Rat galanin(2–29)	87 ± 28 (2)	263.6
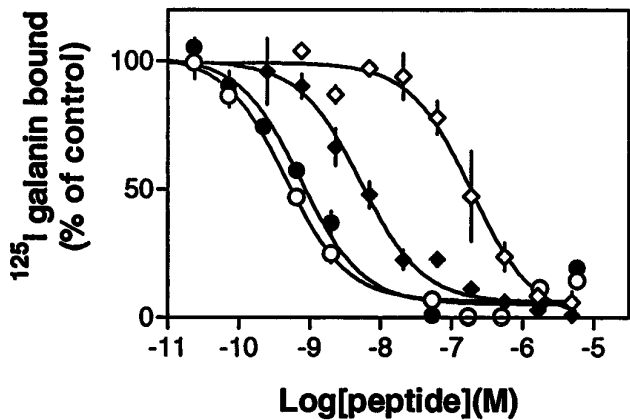
Fig. 5. Competition of ^{125}I-human galanin by galanin(2–29) for rat GalR1 and GalR2 receptors. Data are one representative experiment performed in duplicate in which rat GalR1 and GalR2 receptors from transfected COS-1 cells were directly compared. Symbols shown are rat galanin with GalR1 (○) and GalR2 (●), and galanin(2–29) with GalR1 (◇) and GalR2 (◆).

tion with GalR2 (Fig. 5). Consistent with the suggestion of an interaction with Glu269 of the rat GalR1 receptor is the observation that this residue is not conserved in the rat GalR2 receptor (Fig. 1C).

Rat galanin caused concentration dependent inhibition of forskolin-stimulated cAMP production in COS-1 cells transiently transfected with rat GalR2 cDNA. Nonlinear regression analysis of the data revealed a maximum inhibition of 31 ± 2% and an EC_{50} value of 0.22 ± 0.44 nM (Fig. 6). The effect of galanin was completely blocked by overnight incubation with pertussis toxin (100 ng/ml) before the assay. Therefore, the cloned rat GalR2 receptor is functional in mediating intracellular signaling. Like the rat GalR1 recep-
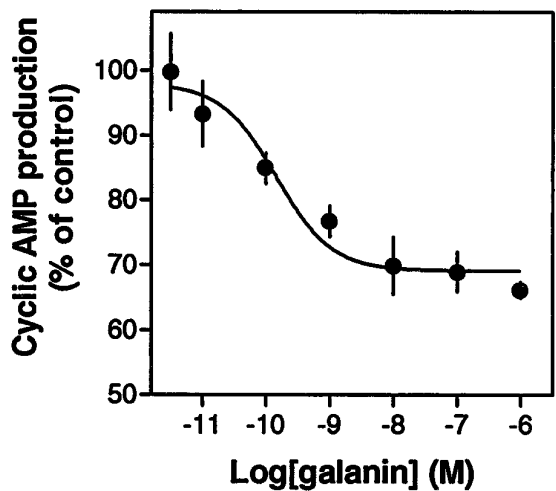

Fig. 6. Inhibition by galanin of forskolin-stimulated cAMP production in rat GalR2 receptor-expressing COS-1 cells. Transfected COS-1 cells were incubated in the presence of 0.1 mM forskolin alone (control) and rat galanin at indicated concentrations. Results shown are mean ± standard deviation from an experiment performed in duplicate. Similar results were obtained in a repeating experiment. *Curve,* best fit to all the data points by one-site nonlinear regression analysis. The basal and forskolin-stimulated intracellular cAMP levels were 55 ± 5 ($n = 4$) and 221 ± 26 ($n = 4$) pmol/10[6] cells, respectively. The basal cAMP level was not affected by incubation with galanin (1 μM).

tor, activation of rat GalR2 receptor also leads to inhibition of forskolin-stimulated cAMP synthesis.

In summary, we have isolated a new galanin receptor subtype and characterized its pharmacological profile using galanin related fragments and chimeric peptides. When compared with the known GalR1 receptor, the new receptor, GalR2, possesses a wide tissue distribution and has a distinct pharmacological profile, characterized by a high affinity for galanin(2–29). A GalR2 receptor has been reported by two other groups. Forray et at reported a receptor that displayed inhibition of forskolin-stimulated cAMP production and similar wide distribution of GalR2 mRNA among central and peripheral tissues (24). A GalR2 receptor with 35% homology to the GalR1 receptor also inhibited forskolin-stimulated cAMP accumulation, and showed expression in brain regions and dorsal root ganglia of the spinal cord (25, 26). However, limited sequence information in these reports does not allow to determine whether these two receptors are identical to each other or to the receptor described in this report. In addition to the unambiguous identification of a new galanin receptor subtype, we have for the first time identified a ligand that differentiates between the two defined galanin receptor subtypes. These results using the two galanin receptor subtypes complement earlier observations of different pharmacological profiles in different tissue and cell preparations. Although galanin(2–29) is not know to be a natural ligand, the GalR2 receptor may mediate functions through variable expressions regulated by its own promoter with different tissue-specificity and strength. Alternatively, natural ligands for GalR2 with pharmacological properties distinct from that of the GalR1 receptor may exist and yet have to be identified. It is possible that more than two galanin receptors exist and account for the diverse physiological functions that galanin mediates. The cloning of GalR2 should aid in the cloning and identification of these putative new gala-

nin receptors. The availability of GalR2 receptor should also provide an additional tool to better define the roles of the galanin receptors in various physiological processes.

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