Communication

Cloning and Expression Characterization of a Novel Galanin Receptor

IDENTIFICATION OF DIFFERENT PHARMACOPHORES WITHIN GALANIN FOR THE THREE GALANIN RECEPTOR SUBTYPES

Suke Wang†, Chaogang He, Tanaz Hashemi, and Marvin Bayne

From the Department of CNS/CV Biological Research, Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Galanin, a 29–30 amino acid neuropeptide, is found in the central and peripheral nervous systems and displays several important physiological activities. The actions are believed to be mediated through distinct G protein-coupled receptors. To date, two galanin receptor subtypes have been cloned. In this report, we describe the cloning and expression of a cDNA encoding a novel galanin receptor (GalR3). The receptor has 370 amino acids and shares 36 and 54% homology with the rat GalR1 and GalR2 receptors.125I-Porcine galanin binds the rat GalR3 receptor expressed in COS-7 cells with high affinity (Kd = 0.6 nm) and could be displaced by galanin and galanin fragments and galanin-chimeric peptides. The pharmacological profile of this novel receptor is distinct from those of GalR1 and GalR2, revealing different pharmacophores within galanin for the three galanin receptor subtypes. Northern blot analysis showed expression in heart, spleen, and testis. Unlike GalR1 and GalR2, no expression of GalR3 was detectable in the brain, suggesting that GalR3 may mediate some of the peripheral functions of galanin.

Galanin is a 29–30 amino acid neuropeptide with no significant homology to any known family of biologically active peptides (1). Galanin is widely distributed in the central and peripheral nervous systems and is highly expressed in various regions of the brain. Many physiological processes are modulated by galanin, including neurotransmitter and hormone release (2), spinal reflexes, nociception (3), firing of noradrenergic neurons, and contraction of gastrointestinal smooth muscle (4, 5). Like neuropeptide Y, centrally administered galanin po-

1 The abbreviations used are: PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; Ga1r, galanin receptor; RACE, rapid amplification of cDNA ends; DAGO, [d-Ala6,N-methyl-

‡ To whom correspondence should be addressed. Fax: 908-298-2383; E-mail: suke.wang@spcorp.com.

PRODUCTS OF THE AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, INC.

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.

This paper is available on line at http://www.jbc.org
The translation of the receptor. Cycling profile of 95 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1 min (40 cycles) was used to obtain the 0.7-kb vector pCR 2.1. A GC melt reagent (CLONTECH) at 10–20% (v/v) of original stock (5 M) was always used in both PCR and RACE reactions.

DNA Sequencing and Analysis—The DNA sequences of clones were determined on both strands using Applied Biosystems Prism dye termination DNA sequencing reagents and an Applied Biosystems 373 automated sequencing apparatus (Perkin-Elmer). DNA and protein sequence comparisons were performed with the DNA* software (DNASTAR Inc., Madison, WI).

Transfection of COS-7 Cells—Rat GalR3 cDNA was introduced into COS-7 cells by electroporation as described previously (15) or by the use of LipofectAMINE method (Life Technologies, Inc.) according to the manufacturer’s instructions. The two methods gave comparable levels of expression.

Receptor Membrane Preparation—Three days following the transfection of the COS-7 cells, receptor membrane was prepared as previously described (15).

125I-Galanin Binding Assay—Binding of 125I-porcine galanin to the membrane preparations was performed as previously described (15) except that 20 µg of membrane protein was used in the saturation and competition binding assays. All data were analyzed by nonlinear regression (Prism, GraphPad, San Diego, CA) and the KI, calculated according to the method of Cheng and Prusoff (17).

Northern Blot Analysis of Rat GalR3—A rat multitissue Northern blot (CLONTECH) was hybridized for 15 h at 65 °C in a ExpressHyb solution (CLONTECH) using 32P-labeled rat GalR3 probe (labeled with a random priming kit, Life Technologies, Inc., specific activity = 3 × 108 cpm/µg). After hybridization, the blot was washed with wash solution I (2 × SSC, 0.05% SDS) for 30 min at room temperature then with wash solution II (0.1 × SSC, 0.1% SDS) for 30 min at room temperature, 1 h at 48 °C, 1 h at 52 °C, and 30 min at 54 °C. The blot was then wrapped with Saran Wrap and exposed to Kodak BioMax films at –80 °C for 1 week. The same blot was stripped and hybridized in a similar manner with a 32P-labeled actin cDNA to ensure loading of poly(A)+ mRNA from the tissues onto the blot.

RESULTS AND DISCUSSION

BLAST search (18) of the GenBank™ data base with the human GalR1 receptor amino acid sequence (10) as query identified a portion of a human genomic clone (accession number Z82241) that possessed high homology with the amino acids 37–132 of human GalR1 (third reading frame of the positive strand, 55% identity). The homology was greater than that between rGalR1 and rGalR2 (40%), suggesting that this human clone may contain a portion of a new galanin receptor.

Several pairs of PCR primers were generated according to the human genomic sequence and used in PCR with cDNA reverse-translated from rat liver RNA as template to obtain rat GalR3 cDNA. A PCR cycling paradigm employing low annealing temperature with two PCR primers, oligonucleotide 177 and AP2 (adapted primer) were used in the primary PCR with a rat brain cDNA library as template, producing a PCR product. The approximately 700-base pair fragment was cloned into vector pCR3.1 and sequenced. Comparison of the DNA sequence with the nucleotide sequences in GenBank™ revealed 86, 65, and 63% identities with the human genomic clone (Z82241), rat GalR2, and human GalR1 receptor amino acid sequence (10) as query identified a portion of a human genomic clone (accession number Z82241), rat GalR2, and human GalR1, respectively. Therefore, the rat clone appeared to be a portion of a novel rat galanin receptor (Fig. 1A). A PCR cycling paradigm employing low annealing temperature with two PCR primers, oligonucleotide 93C and oligonucleotide 120B, produced a PCR product. The approximately 700-base pair fragment was cloned into vector pCR3.1 and sequenced. Comparison of the DNA sequence with the nucleotide sequences in GenBank™ revealed 86, 65, and 63% identities with the human genomic clone (Z82241), rat GalR2, and human GalR1, respectively. Therefore, the rat clone appeared to be a portion of a novel rat galanin receptor (Fig. 1A).

RACE and PCR sib selection were performed to extend the cDNA sequence toward the 5’ and 3’ directions. In RACE, primers oligo172 and AP1 (outer adaptor primer) were used in the primary PCR with a rat brain cDNA library as template and primers oligo177 and AP2 (inner adaptor primer) were used in the secondary PCR with product of the primary PCR as template. The approximately 700-base pair fragment was cloned into vector pCR3.1 and sequenced. Comparison of the DNA sequence with the nucleotide sequences in GenBank™ revealed 86, 65, and 63% identities with the human genomic clone (Z82241), rat GalR2, and human GalR1, respectively. Therefore, the rat clone appeared to be a portion of a novel rat galanin receptor (Fig. 1A).

RACE and PCR sib selection were performed to extend the cDNA sequence toward the 5’ and 3’ directions. In RACE, primers oligo172 and AP1 (outer adaptor primer) were used in the primary PCR with a rat brain cDNA library as template and primers oligo177 and AP2 (inner adaptor primer) were used in the secondary PCR with product of the primary PCR as template. The approximately 700-base pair fragment was cloned into vector pCR3.1 and sequenced. Comparison of the DNA sequence with the nucleotide sequences in GenBank™ revealed 86, 65, and 63% identities with the human genomic clone (Z82241), rat GalR2, and human GalR1, respectively. Therefore, the rat clone appeared to be a portion of a novel rat galanin receptor (Fig. 1A).

RESULTS AND DISCUSSION

BLAST search (18) of the GenBank™ data base with the human GalR1 receptor amino acid sequence (10) as query identified a portion of a human genomic clone (accession number Z82241) that possessed high homology with the amino acids 37–132 of human GalR1 (third reading frame of the positive strand, 55% identity). The homology was greater than that between rGalR1 and rGalR2 (40%), suggesting that this human clone may contain a portion of a new galanin receptor.

Several pairs of PCR primers were generated according to the human genomic sequence and used in PCR with cDNA reverse-translated from rat liver RNA as a template to obtain rat GalR3 cDNA (Fig. 1A). A PCR cycling paradigm employing low annealing temperature with two PCR primers, oligonucleotide 93C and oligonucleotide 120B, produced a PCR product. The approximately 700-base pair fragment was cloned into vector pCR3.1 and sequenced. Comparison of the DNA sequence with the nucleotide sequences in GenBank™ revealed 86, 65, and 63% identities with the human genomic clone (Z82241), rat GalR2, and human GalR1, respectively. Therefore, the rat clone appeared to be a portion of a novel rat galanin receptor (Fig. 1A).
Fig. 2. Tissue distribution of rat GalR3 by Northern blot analysis. Northern blot containing rat poly(A)+ mRNA was hybridized with a 32P-labeled the 700-base pair rat GalR3 cDNA sequence (the cloned PCR fragment with primers 93C and 120B, Fig. 1A). 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 32P-labeled β-actin cDNA to ensure comparable loading of mRNA from the tissues (bottom).

Fig. 3. Saturation analysis of 125I-porcine galanin (125I-pGal) binding to membranes prepared from transfected COS-7 cells with pcDNA3-rGalR3 (clone A5-3). Data shown represent the specific binding at 0.3 nM, which is the Ki of galanin (Fig. 1C). It was determined by Scatchard analysis (17) determined by 

| Ligand                                   | Kd (nM) | Kd/ki (nM) |
|------------------------------------------|---------|------------|
| Rat galanin-(1–29)                       | 1.47 ± 0.42 | 7 ± 1 |
| C7 (galanin-(1–13)-spantide I)           | 0.75 ± 0.06 | 2 ± 1 |
| Rat galanin-(1–19)                       | 6.25 ± 0.81 | 2 ± 1 |
| M35 (galanin-(1–13)-bradykinin(2–9))     | 7.50 ± 2.95 | 4 ± 1 |
| Rat galanin-(2–29)                       | 12.6 ± 6.69 | 8 ± 1 |
| [Ala6,D-Trp8]Galamin-(1–15)-ol            | 17.9 ± 8.64 | 12 ± 1 |
| Rat galanin-(1–16)                       | 49.6 ± 15.3 | 33 ± 1 |
| M40                                      | 54 ± 23.7  | 36 ± 1 |
| M15 (galantide or gal-(1–13)-SP(5–11))   | 85 ± 52.4  | 58 ± 1 |
| Rat galanin-(3–29)                       | >1000 (3)  | >650 |
| Rat galanin-(10–29)                      | >1000 (2)  | >650 |
| Preprogalanin-(1–30)                     | >1000 (2)  | >650 |
| [α-Thr4,β-Pro3]Galamin-(1–15)-ol          | >1000 (2)  | >650 |
| GMAP-(1–14)                              | >1000 (2)  | >650 |
| GMAP-(44–59)                             | >1000 (3)  | >650 |
| Rat galR2 receptor                       | 1.48 ± 0.59 | 5 ± 1 |
| M15                                      | 1.11 ± 0.16 | 0.75 |
| M40                                      | 1.92 ± 0.92 | 1.30 |
| Rat galanin-(1–19)                       | 2.88 ± 1.48 | 1.95 |
| Rat galanin-(1–16)                       | 4.7 ± 2.0 (3)| 3.18 |
| Rat galanin-(3–29)                       | 5.66 ± 3.7 (2)| 3.85 |
| >1000 (4)                                | >650 |
| GMAP-(44–59)                             | >1000 (2)  | >650 |
| Rat galR1 receptor                       | 0.98 ± 0.22 (3)| 1 |
| Rat galanin-(1–16)                       | 4.8 ± 1.5 (2)| 4.9 |
| Rat galanin-(2–29)                       | 85 ± 12 (3)| 87 |
| >1000 (2)                                | >1020 |

Alignment with rat GalR2 displayed a generally higher homology in the TM1s with rat GalR1, with the highest in TM2, TM3, and TM4, being 92, 83, and 71% identical, respectively. The N terminus and the C terminus possess least homology with both rat GalR1 and rat GalR2 receptors (Fig. 1C).

Northern blot analysis was performed to examine the tissue distribution of GalR3 mRNA. A single band of ~3.5 and ~3 kb was detected in heart and testis, respectively (Fig. 2). There was a strong, broad band at higher molecular weight (5–8 kb) in spleen and testis, indicating a heterogeneous population of the transcript in these tissues. There was no significant expression in kidney, skeletal muscle, liver, brain, or lung. The low abundance of GalR3 in the brain is contrasted to the distribution of GalR1, which is significantly expressed only in brain and spinal cord (12, 13), and to that of GalR2, which is expressed in both central and peripheral tissues (14, 15). Given that the GalR3 cDNA was cloned from a hypothalamus library, it seems likely that there is a low level of expression of GalR3 in this part of the brain. The overall expression pattern of GalR3 suggests that the receptor may mediate galanin functions in the cardiovascular system (22, 23) and in sexual behavior (24, 25).
vector alone was negligible. The specific binding was saturable at high affinity, showing a $K_D$ value for $^{125}$I-galanin of 0.55 ± 0.15 nM and a $B_{max}$ of 28.1 ± 1.1 fmol/mg of membrane protein (mean ± S.D. from three independent transfections). A representative binding curve is illustrated in Fig. 3. No specific binding was observed when $^{125}$I-labeled somatostatin was used in the binding assays.

The ability of several galanin antagonists and agonists to bind GalR3 were examined in radioligand competition binding assays. $^{125}$I-porcine galanin binding to membranes prepared from COS-7 cells transfected with pcDNA3-rGalR3 cDNA could be displaced with galanin fragments and galanin-related chimeric peptides (Table I). Galanin, C7, galanin-(1–19), M35, and galanin-(2–29) bound GalR3 with high affinity (within 9-fold $K_i$ of galanin), while M40, galanin-(1–16), and M15 bound rGalR3 at high affinity, showing a $K_D$ vector alone was negligible. The specific binding was saturable with relatively lower affinities (33–60 times $K_i$ of galanin), while M40, galanin-(1–16), and M15 bound rGalR3 with relatively lower affinities (33–60 times $K_i$ of galanin) (Table I). No binding was detected in the assays with galanin-(2–29) and GalR3 receptor subtypes.

Asn in galanin, Gly1 of galanin is not critical for galanin to bind GalR3, whereas the residue is important for binding of galanin to GalR1 (15). In contrast, galanin-(3–29) did not bind either GalR1, GalR2, or GalR3 (Table I), indicating a crucial role of Trp2 of galanin in binding of galanin to all three receptor subtypes.

In summary, we have cloned and characterized a novel galanin receptor subtype termed GalR3. The receptor shares homology (36–54%) to the previously cloned GalR1 and GalR2 receptors. The distribution of GalR3 mRNA expression is strikingly different from those of GalR1 and GalR2 and appears to be restricted to the peripheral tissues. The pharmacology of the GalR3 receptor is distinguished from the other two receptors by the requirement of amino acids 17–19 of galanin (Fig. 4). The three galanin receptor subtypes show different pharmacological profiles with respect to galanin analogs, suggesting that they bind to distinct pharmacophores within the galanin peptide (Fig. 4B). This observation suggests the potential for physiological control of galanin receptor subtype activation by selective ligand modification such as differential proteolysis (27, 28). The characterization of this new galanin receptor should aid in delineating specific physiological roles of the galanin receptor subtypes.

Acknowledgments—We thank Drs. Michael Graziano and Catherine Strader for critical reading of the manuscript.

REFERENCES

1. Tatemoto, K., Rokaeus, A., Jornwall, H., McDonald, T. J., and Mutt, V. (1983) FEBS Lett. 164, 124–128.
2. Dunning, B. E., Ahren, B., Veith, R. C., Bottcher, G., Sundler, F., and Taborsky, G. J. (1986) Am. J. Physiol. 251, E127–E133.
3. Verge, V. M. K., Xu, X.-J., Langel, U., Hokfelt, T., Wiesenfeld, Z., and Bartfai, T. (1993) Neurosci. Lett. 149, 193–197.
4. Ekblad, E., Jakanian, R., Sundler, F., and Wahlestedt, C. (1985) Br. J. Pharmacol. 86, 241–246.
5. Crawley, J. N. (1995) Regul. Pept. 59, 1–16.
6. Crawley, J. N., Austin, M. C., Fisone, G., M15, Langel, U., Fisone, G., and Bartfai, T. (1990) J. Neurochem. 52, 3659–3700.
7. Rossowski, W. J., Rossowski, T. M., Zacharia, S., Ertan, A., and Cey, H. H. (1990) Peptides 11, 333–338.
8. Fisone, G., Berthold, M., Bedecs, K., Uden, A., Bartfai, T., Bertorelli, R., Consolo, S., Crawley, J., Martin, B., Nilsson, S., and Hokfelt, T. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8589–8591.
9. Wynick, D., Smith, D. H., Akinsanya, K., Bhogal, R., Purkiss, P., Yanaihara, N., and Bloom, E. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4231–4235.
10. Habert-Ortoli, E., Amirabnoff, B., Loquet, I., Laburthe, M., and Mayux, J. F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9780–9783.
11. Burgevin, C. M., Loquet, I., Queraronet, D., and Habert-Ortoli, E. (1995) J. Mol. Neurosci. 6, 33–41.
12. Parker, E. M., Izzarelli, D. G., Nowak, H. P., Iben, L. G., Wang, J., and Goldstein, M. E. (1995) Mol. Brain Res. 34, 179–189.
13. Wang, S., He, C., Maguire, M., Clemmons, A., Burrier, R., Gzuzi, M., Strader, C., Parker, E., and Bayne, M. (1997) FEBS Lett. 411, 225–230.
14. Howard, A. D., Tan, C., Shiao, L.-L., Palyha, O. C., McKee, K. K., Weiger, D., and Sullivan, K. A. (1997) FEBS Lett. 405, 285–290.
15. Wang, S., Hashemi, T., He, C., Strader, C., and Bayne, M. (1997) Mol. Pharmacol. 52, 337–343.
16. Probst, W. C., Snyder, L. A., Schuster, D. I., Broius, J., and Sealfon, S. C. (1992) DNA Cell Biol. 11, 1–20.
17. Cheng, Y., and Prusoff, W. H. (1973) Biochem. Pharmacol. 22, 3099–3108.
18. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410.
19. Hein, J. (1990) Methods Enzymol. 183, 626–645.
20. Bruno, J. F., Xu, Y., Song, J., and Berelowitz, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11151–11155.
21. Fukuda, K., Kato, S., Mori, K., Nishi, M., and Takeshima, H. (1993) FEBS Lett. 327, 311–314.
22. Ulman, L. G., Evans, H. F., Iismaa, T. P., Potter, E. K., McCloskey, D. I., and Shime, (1992) Neuropeptides 16, 105–108.
23. Ulman, L. G., Potter, E. K., and McCloskey, D. I. (1994) Regul. Pept. 51, 17–23.
24. Poggio, R., Rasson, E., and Bertolini, A. (1992) Eur. J. Pharmacol. 213, 87–90.
25. Benelli, A., Arletti, R., Bertolini, A., Mesozzi, B., Basaglia, R., and Poggio, R. (1994) Eur. J. Pharmacol. 260, 279–282.
26. Kakuymaya, H., Mochizuki, T., Iuchi, K., Yamabe, K., Hosoe, H., Hoshino, M., and Yamafara, N. (1997) Biomed. Res. 18, 49–56.
27. Bedecs, K., Langel, U., and Bartfai, T. (1995) Neuropeptides 29, 137–143.
28. Jureus, A., Lindgren, M., Langel, U., and Bartfai, T. (1997) in Seventh Annual Summer Neuropeptide Conference, Key West, FL, June 22–26, 1997, International Neuropeptide Society.