Differential Intracellular Signaling of the GalR1 and GalR2 Galanin Receptor Subtypes

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Received November 19, 1997; Revised Manuscript Received February 24, 1998

ABSTRACT: The diverse physiological functions exerted by the neuropeptide galanin may be regulated by multiple G protein-coupled receptor subtypes and intracellular signaling pathways. Three galanin receptor subtypes (GalRs) have been recently cloned, but the G protein coupling profiles of these receptors are not completely understood. We have generated GalR1- and GalR2-expressing Chinese hamster ovary (CHO) cell lines and systematically examined the potential for these two receptors to couple to the Gs, Gi, Go, and Gq proteins. Galanin did not stimulate an increase in cAMP levels in GalR1/CHO or GalR2/CHO cells, suggesting an inability of either receptor to couple to Gs. Galanin inhibited forskolin-stimulated cAMP production in GalR1/CHO cells by 70% and in GalR2/CHO cells by 30%, suggesting a strong coupling of GalR1 to Gi and a more modest coupling between GalR2 and Gi. GalR1 and GalR2 both mediated pertussis toxin-sensitive MAPK activity (2−3-fold). The stimulation mediated by GalR1 was inhibited by expression of the C-terminus of β-adrenergic receptor kinase (βARKct), which specifically inhibits G$\beta\gamma$ signaling, but was not affected by the protein kinase C (PKC) inhibitor, bis[indolylmaleimide], or cellular depletion of PKC. In contrast, GalR2-mediated MAPK activation was not affected by βARKct expression but was abolished by inhibition of PKC activity. The data demonstrate that GalR1 is coupled to a Gi$\beta\gamma$ signaling pathway to mediate MAPK activation. In contrast, GalR2 utilizes a distinct signaling pathway to mediate MAPK activation, which is consistent with Go-mediated MAPK activation in CHO cells. Galanin was unable to stimulate inositol phosphate (IP) accumulation in CHO or COS-7 cells expressing GalR1. In contrast, galanin stimulated a 7-fold increase in IP production in CHO or COS-7 cells expressing GalR2. The GalR2-mediated IP production was not affected by pertussis toxin, suggesting a linkage of GalR2 with Gq/G11. Thus, the GalR1 receptor appears to activate only the Gi pathway. By contrast, GalR2 is capable of stimulating signaling which is consistent with activation of Go, Gq/G11, and Gi. The differential signaling profiles and the tissue distribution patterns of GalR1 and GalR2 may underlie the functional spectra of galanin action mediated by these galanin receptors and regulate the diverse physiological functions of galanin.

Since the discovery of the neuropeptide galanin (29−30 amino acids) from the bovine digestive system (*1*), numerous studies have shown that galanin mediates a variety of physiological activities (*2*−*4*). Initial studies revealed galanin functions in control of release of gastrin and stomach acid and in modulation of contraction of gastrointestinal smooth muscles (*5*−*8*). Galanin was then found to be widely distributed and abundantly expressed in both peripheral and central tissues, which led to studies demonstrating a variety of galanin actions in the peripheral and central nervous systems, including neurotransmitter and hormone release, firing of noradrenergic neurons, spinal reflexes, nociception, and stimulation of food intake (*2*, *3*, *9*). Recent evidence also suggests that galanin possesses mitogenic and neurotrophic roles important for the development of the nervous system (*10*) and for recovery from neuronal injury (*11*).

The actions of galanin are mediated by specific galanin receptors. Molecular cloning efforts initially identified two galanin receptors (GalR1 and GalR2)[1] (*12*−*15*). Both receptors belong to the G protein-coupled receptor superfamily, characterized by seven transmembrane domains. The amino acid sequences of GalR1 and GalR2 are significantly different from one another, sharing only 40% homology (*14*). Both receptors bind to [^{125}I]galanin with high affinity (0.3−0.5 nM), and the binding of the radioligand can be displaced by various galanin fragments and galanin-related chimeric peptides. The two receptors, however, can be pharmacologically distinguished by galanin(2−29), a galanin fragment missing the N-terminal Gly residue of galanin which binds preferentially to GalR2 (*14*). Northern blot and in situ hybridization studies demonstrated that GalR1 is significantly expressed only in brain and spinal cord (*12*, *16*), while the expression of the GalR2 receptor is abundant and widespread in both central and peripheral systems (*13*, *14*). More

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[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; FCS, fetal calf serum; GalR, galanin receptor; PCR, polymerase chain reaction; MAPK, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; M1AChR, the m1 muscarinic acetylcholine receptor; PTX, pertussis toxin; LPA, lysophosphatidic acid; IP, inositol phosphate; βARKct, C-terminus of the β2-adrenergic receptor kinase; PKC, protein kinase C.

S0006-2960(97)02840-7 CCC: $15.00
Published on Web 04/22/1998

recently, we have cloned a third galanin receptor subtype, GalR3 (*17*). The amino acid sequence of GalR3 is significantly different from those of GalR1 and GalR2, sharing only 36% and 54% homology with the two receptors, respectively. The expression of this new subtype appears to be restricted to peripheral tissues (*17*).

There are several families of G proteins that are regulated by G protein-coupled receptors. Activation of Gs results in increased adenylyl cyclase I activity and subsequent cAMP production. In contrast, activation of Gi inhibits adenylyl cyclase I. Gi activation is also coupled to activation of adenylyl cyclase II (*18, 19*), phospholipase A_2 (*20*), K^+ channels (*21*−*23*), phosphoinositide-3-kinase (*24*), and specific isoforms of phospholipase C (*25*). Activation of Gq results in increased phospholipase C activity and subsequent inositol phosphate (IP) production. Gi, Gq, and Go are also capable of mediating activation of mitogen activated protein kinase (MAPK). MAPKs are serine/threonine kinases that phosphorylate and activate numerous transcription factors involved in cell growth and proliferation through a complex pathway which is currently being elucidated (*26*). Gi-coupled receptors mediate MAPK activation via a signaling pathway that utilizes the $\beta\gamma$-subunit of Gi, PI-3-kinase γ, the protein kinase Src, the phosphoprotein Shc, the adapter protein Grb2, and the Ras-GTP exchange factor Sos (*24, 27*−*33*). Sos activates the small molecular weight GTP-binding protein Ras, which activates a series of kinases including Raf, MEK (mitogen-activated protein kinase/erk kinase), and finally MAPK (*20, 34*−*38*). The Gi-mediated pathway is sensitive to inhibition by pertussis toxin (PTX) and is independent of PKC activity. The Gq-mediated MAPK signaling pathway utilizes the α-subunit of Gq, is insensitive to PTX, and is dependent on PKC activity (*28*). The Go-mediated MAPK signaling pathway employs the α-subunit of Go, is sensitive to PTX, and is dependent on PKC activity (*39*).

A complete understanding of the actions of galanin requires identification and functional characterization of galanin receptor subtypes. One important biochemical aspect of the receptors is to define all the signaling pathways that provoke a range of the cellular responses to the binding of galanin to the receptors. Although it has been reported that GalR1 mediates galanin-stimulated inhibition of forskolin-evoked cAMP production (*12*) and GalR2 mediates galanin-stimulated inositol phosphate accumulation (*40*), a thorough analysis of the ability of GalR1 and GalR2 to couple to various G protein subtypes has not been reported. In the present study, the effects of galanin or galanin-derived peptides on cAMP production, MAPK activity, and IP accumulation in CHO cell lines expressing the rat GalR1 or GalR2 receptor were determined, thereby assessing the ability of GalR1 and GalR2 to couple to four major classes of G proteins: Gs, Gi, Go, and Gq. The results show that the G protein coupling profiles of the two receptors are distinct. GalR1 couples only to Gi, while GalR2 couples to Gi, Go, and Gq.

MATERIALS AND METHODS

[^{125}I]Porcine galanin (2200 Ci/mmol) and cAMP Flash-Plates were purchased from DuPont-NEN (Boston, MA). Lipofectamine transfection agent and oligonucleotides used in this study were purchased or custom-synthesized by BRL Life Technologies (Grand Island, NY). Rat galanin was purchased from Peninsula Laboratories (Belmont, CA). Rat galanin(2−29), and rat galanin(3−29) were custom-synthesized by Bio-synthesis, Inc. (Lewisville, TX).

Generation of CHO Cell Lines Stably Expressing the Rat GalR1 and GalR2 Galanin Receptors. The cDNA encoding rat GalR1 receptor was generated by RT-PCR with total RNA prepared from rat hypothalamus. The 5′ and 3′ primers designed for the PCR were based on the published rat GalR1 cDNA sequence (*12*). The full-length rat GalR1 cDNA was then cloned into vector pcDNA3 (Invitrogen). The rat GalR2 was obtained and cloned in expression vector pCR3.1 (Invitrogen) as previously described (GenBank accession no. Y15248) (*14*). The DNA sequences of clones were determined on both strands using ABI Prism dye termination DNA sequencing reagents and an ABI 373 automated sequencing apparatus (Perkin-Elmer, Branchburg, NJ). DNA and protein sequence comparisons were performed with the DNA* software (DNAstar Inc. Madison, WI). Stable cell lines that express either the GalR1 or the GalR2 receptor were obtained by transfection of CHO cells by the electroporation method as described previously (*16*). The transfected cells were diluted 1:10 000 in 100-mm plates, and individual clones were isolated in the presence of 500 μg/mL G418 (geneticin). When individual colonies were grown to ∼1−2 mm in diameter, cells in each clone were transferred into 96-well plates. After the cells were expanded and grown in 24-well plates, [^{125}I]porcine galanin binding was performed with the intact cells and 4−6 clones from each receptor with highest bound counts were further expanded and characterized. Nontransfected CHO cells were grown in F12 medium supplemented with 10% FCS (v/v). Transfected CHO cells and cells from the selected stable expression clones were grown in the same medium plus 500 mg/mL G418.

Receptor Membrane Preparation and Radioligand Binding Assay. Cell medium was removed, and the cells were washed three times with PBS (without Ca^{2+}/Mg^{2+}). Hepes buffer (5 mL, 5 mM), pH 7.4, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.1 mg/mL bacitracin were added to the plate and incubated for 15 min at room temperature. The cells were scraped off the plates and centrifuged at 13 000*g* for 15 min at 4 °C. The cell pellet was resuspended in 2 mL of 25 mM Tris-Cl, pH 7.4, and 0.2 mM PMSF by vortexing and dispersed with a syringe attached to a 23-gauge needle. Protein concentrations were determined by the BCA method (Pierce, Rockford, IL). Ligand saturation binding of [^{125}I]porcine galanin to the membrane preparations was performed in 200 μL of binding buffer (25 mM Tris-Cl (pH 7.4), 1% bovine serum albumin (w/v), 0.1% bacitracin, 2 μg/mL leupeptin, 0.1 mM PMSF, and 10 mM $MgCl_2$). Incubations were at room temperature for 1 h and were terminated by rapid vacuum filtration through Multiscreen FB Filter Plates (Millipore, Bedford, MA) which had been pretreated with 0.3% polyethylenimine. The filters were then washed three times with 100 μL of phosphate-buffered saline (pH 7.4). Nonspecific binding was determined in the presence of 5 mM rat galanin. All data were analyzed by nonlinear regression (Prism, GraphPad, San Diego, CA), and the K_i was calculated according to the method previously described (*41*).

Cyclic AMP Determination. CHO cells expressing the rat GalR1 or GalR2 receptors were seeded in 96-well plates at a density of 5×10^4 cells/well. After 24 h of growth, the cells were gently rinsed with an incubation buffer (Hanks solution with divalent cations, 10 mM Hepes (pH 7.4), and 0.2%BSA (w/v)) and incubated for 15 min at room temperature in 200 μL of the incubation buffer. The buffer was removed, and the cells were incubated with 200 μL of the incubation buffer containing appropriate concentrations of galanin peptides, 0.2 mM 3-isobutyl-1-methylxanthene (IBMX), and 0.1 mM forskolin for 45 min at room temperature. The buffer was removed, and the cells were washed with 200 μL of the incubation buffer. The cells were lysed by incubation with 75 μL of ethanol at room temperature for 20 min. The ethanol was evaporated at 70 °C to dryness, and the dried cell lysate was dissolved in 200 μL of cAMP assay buffer (DuPont-NEN), 100 μL of which was used in determination of cAMP concentrations. The content of cAMP in the cell lysates was determined by use of the cAMP FlashPlate method (DuPont-NEN). IC_{50} and maximum inhibition of the forskolin-stimulated cAMP production were determined by nonlinear regression analysis.

MAPK Activity Assay. Cells were split into 12-well plates and incubated for 1−2 days. Cells were then serum-deprived overnight in growth media containing 0.5% FBS, incubated with agonists as indicated for 5 min at 37 °C, washed with 1 mL of PBS (4 °C), and lysed at 4 °C with 0.1 mL of RIPA buffer (50 mM Tris-Cl (pH 8), 150 mM NaCl, 1% NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% SDS, 10 mM NaF, and 10 mM Na pyrophosphate). Cell lysates were centrifuged at 16000*g* at 4 °C for 15 min, and the supernatants were collected. MAPK activity was measured using the Amersham p42/p44 MAPK enzyme assay system. Cell lysates (15 μL) were incubated with MAPK-specific peptide substrate and [γ-^{32}P]ATP for 30 min at 30 °C. The reaction mixtures were then transferred to phosphocellulose paper, and the paper was washed twice with 1% acetic acid and twice with water. ^{32}P incorporation was then measured by liquid scintillation spectroscopy. Coexpression of βARKct with the GalR receptors in CHO cells was achieved by transiently transfecting GalR1/CHO or GalR2/CHO cells with cDNA encoding the C-terminus of β-adrenergic receptor kinase 1 (βARKct). Transfection was performed using the Lipofectamine method (BRL-Life Technology, Gaithersburg, MD). Cells grown in 12-well plates at 80% confluency were washed with F12 medium and incubated at 37 °C for 2 h in 1 mL of F12 medium containing 1 μg/mL plasmid DNA, pRK5 vector alone or pRK5-βARKct, plus 6 μL of Lipofectamine. The solution was aspirated, and 2 mL of F12 medium containing 10% FBS was added. Cells were incubated for 24 h and serum-deprived overnight prior to the MAPK assay as described above.

Inositol Phosphate Turnover Assay. Cells grown in 6-well plates were equilibrated for 24 h in 1−2 μCi/mL myo[^{3}H]-inositol (NEN:NET114A) in regular growth medium. The cells were washed with PBS at 37 °C followed by stimulation for 60 min at 37 °C with agonists in PBS containing 20 mM LiCl and 1 mM $CaCl_2$. The reaction was terminated by removing the medium and adding 1 mL of 0.4 M perchloric acid to each well. The cells were then incubated at 4 °C for 10 min. The solution was removed, and 500 μL of neutralizing solution containing 0.72 M KOH/0.6M $KHCO_3$ was added. A Dowex anion-exchange column was prepared using 1 mL of a 50:50 slurry of Dowex (formate form). One milliliter of supernatant and 3 mL of water was applied to each column. The column was then washed twice with 10 mL of water. IPs were eluted using 3.5 mL of 0.1 M formic acid/1 M ammonium formate and counted using liquid scintillation spectroscopy.

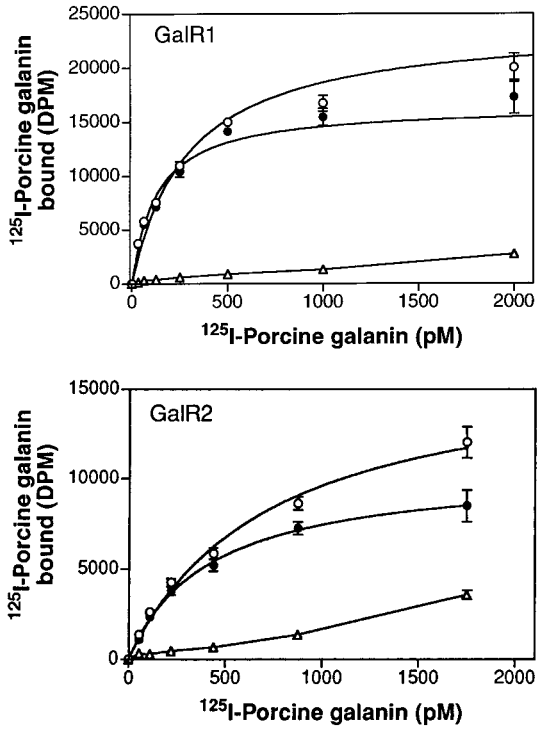

FIGURE 1: Expression of GalR1 and GalR2 in CHO cells. Saturation binding of [^{125}I]porcine galanin was performed with membranes (10 μg for GalR1 and 40 μg for GalR2) prepared from CHO cells expressing rat GalR1 (top) and GalR2 (bottom). Curves represent total binding (○), nonspecific binding (△), and specific binding calculated as the difference between the total and nonspecific binding (●) (mean ± SE, $n = 3$). The nonspecific binding was defined by including 5 μM unlabeled rat galanin in the assays.

RESULTS

Expression of GalR1 and GalR2 in CHO Cells. To obtain stable CHO cell lines that produce the rat GalR1 and GalR2 galanin receptors, CHO cells were transfected with plasmids containing cDNAs encoding GalR1 (GalR1/CHO) or GalR2 (GalR2/CHO) receptor. Cell lines were selected by growing the transfected cells in the presence of G418. Since signal transduction events may depend on the expression levels of the activating receptors (*42*), the level of receptor expression in each of the cell lines was determined by saturation binding of [^{125}I]porcine galanin to membrane preparations from the cell lines (Figure 1). Nonlinear regression analysis of the specific binding data yielded K_d values of 0.13 ± 0.03 and 0.45 ± 0.12 nM and B_{max} values of 339 ± 18 and 55 ± 13 fmol/mg of membrane protein (mean ± SE, $n = 3$) for GalR1 and GalR2, respectively. Thus, when expressed in CHO cells, both GalR1 and GalR2 possessed high affinity for the radioligand and the expression levels for the receptors were within an order of magnitude.

Effect of Galanin on Intracellular cAMP Accumulation. The capabilities of GalR1 and GalR2 to couple to Gs and

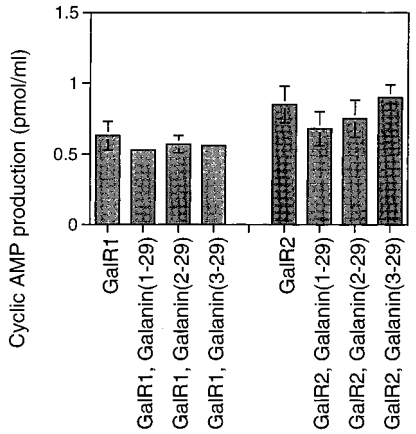

FIGURE 2: Effect of galanin peptides on basal intracellular cAMP production. Cell lysates (100 μL) from rGalR1/CHO or rGalR2/CHO cells were assayed for cAMP concentration. Data are representative of two independent experiments performed in duplicate.

Gi were examined by measuring intracellular cAMP accumulation. To assess Gs coupling, the effects of 3 μM galanin(1−29), galanin(2−29), and galanin(3−29) on the level of intracellular cAMP in GalR1/CHO and GalR2/CHO cells were determined in the presence of a cAMP phosphodiesterase inhibitor, IBMX (0.2 mM). The basal intracellular cAMP level (without galanin peptides) was 0.6−1.0 pmol/mL (Figure 2). Inclusion of 3 μM galanin peptides in the incubation did not cause a significant increase of the cAMP levels in either GalR1/CHO or GalR2/CHO cells (Figure 2). Stimulation with 0.1 mM forskolin, by comparison, resulted in a 40−60-fold increase in intracellular cAMP (data not shown). These data suggest a lack of positive coupling of GalR1 and GalR2 to Gs.

To study the ability of GalR1 and GalR2 to couple to Gi, the effects of the galanin peptides galanin(1−29), galanin(2−29) and galanin(3−29) on forskolin-stimulated cAMP production in GalR1/CHO and GalR2/CHO cells were determined (Figure 3). Galanin(1−29) binds to and activates both GalR1 and GalR2. Galanin(2−29) preferentially binds to and activates GalR2 compared to GalR1. Galanin(3−29) is unable to activate either GalR1 or GalR2. In GalR1/CHO cells galanin(1−29) potently inhibited forskolin-stimulated cAMP production ($IC_{50} = 0.46 \pm 0.15$ nM, and $I_{max} = 75.3 \pm 3.8\%$) (Figure 3A). Galanin(2−29) inhibited the cAMP production only at high concentrations (0.1 and 3.0 μM) with an estimated IC_{50} of 360 ± 206 nM, more than 2 orders of magnitude less potent than galanin(1−29). Galanin(3−29) did not cause any apparent inhibition of forskolin-stimulated cAMP production. In GalR2/CHO cells, both galanin(1−29) and galanin(2−29) potently inhibited forskolin-stimulated cAMP production ($IC_{50} = 1.4 \pm 1.2$ and 5.3 ± 3.7 nM, respectively) (Figure 3B). The maximum inhibition caused by galanin(1−29) and galanin(2−29) was 32 ± 5% and 24 ± 6%, respectively, approximately one-third of that for galanin(1−29) in GalR1/CHO cells (Figure 3). As in GalR1/CHO cells, galanin(3−29) did not cause any inhibition of forskolin-stimulated cAMP production. These data suggest that the GalR1 receptor is more efficiently coupled to Gi compared to GalR2 receptor.

The ability of galanin to stimulate MAPK activity in CHO cells expressing either GalR1 or GalR2 was also examined.

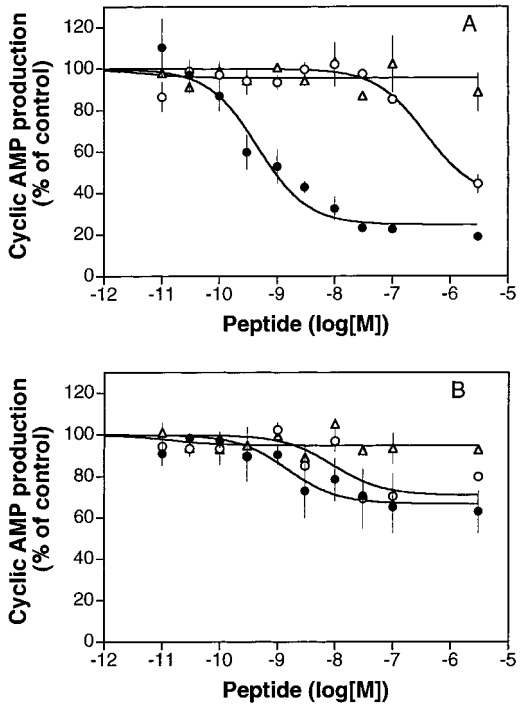

FIGURE 3: Inhibition of forskolin-stimulated cAMP production in GalR1/CHO cells and GalR2/CHO cells. Cyclic AMP in 100 μL of cell lysates of GalR1/CHO cells (A) or GalR2/CHO cells (B) were assayed, and data are expressed as percent of control (no peptide added) cAMP production. Symbols denote galanin(1−29) (●), galanin(2−29) (○), and galanin(3−29) (△). The basal and forskolin-stimulated levels of cAMP in GalR1/CHO cells were 0.58 ± 0.04 ($n = 6$) and 32 ± 5.5 (mean ± SE, $n = 12$) pmol/mL, respectively. The basal and forskolin-stimulated levels of cAMP in GalR2/CHO cells were 0.88 ± 0.28 ($n = 6$) and 38.7 ± 1.6 (mean ± SE, $n = 6$) pmol/mL, respectively.

In GalR1/CHO cells, the presence of 100 nM galanin stimulated MAPK activity to nearly 3 times that of basal level (Figure 4A). Pretreatment of the cells with 100 ng/mL pertussis toxin (PTX) completely blocked the MAPK activity produced by galanin in GalR1/CHO cells. Similarly, PTX blocked the activation of MAPK by lysophophatidic acid (LPA), which is known to mediate MAPK by stimulating a Gi signaling pathway (*34*, *43*). In contrast, MAPK activity provoked by directly activating protein kinase C (PKC) with PMA was not affected by PTX pretreatment. Inhibition of PKC activity, either by pretreatment of the cells with 1 μM bis[indolylmaleimide I] or by depletion of cellular PKC by an overnight incubation with 1 μM PMA, did not affect galanin-stimulated MAPK activity in GalR1/CHO cells (Figure 4A). Stimulation of MAPK by activation of the Gi-coupled LPA receptor was also unaffected by inhibition of PKC activity, while PMA-stimulated MAPK activation was abolished by bis[indolylmaleimide I] or by cellular PKC depletion. These data indicate that GalR1 mediates MAPK activation via a signaling pathway that utilizes a PTX-sensitive G protein and is independent of PKC.

Galanin also stimulated MAPK activity in GalR2/CHO cells (2-fold over control, Figure 4B). Pretreatment of the cells with PTX totally inhibited galanin-stimulated MAPK activity (Figure 4B). However, unlike GalR1/CHO cells, the galanin-stimulated MAPK activity in GalR2/CHO cells was completely inhibited by bis[indolylmaleimide I] or PKC depletion (Figure 4B). LPA and PMA produced the same signaling profile in GalR2/CHO cells compared to GalR1/

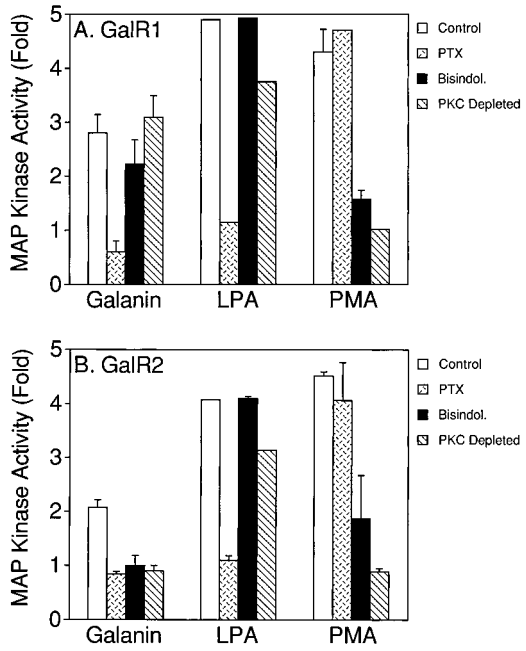

FIGURE 4: Activation of MAPK activity by galanin in GalR1/CHO and GalR2/CHO cells. GalR1/CHO (A) or GalR2/CHO (B) cells were grown in the absence of serum for 16 h and incubated for 5 min at 37 °C in the presence of 100 nM galanin, 10 μM LPA or 1 μM PMA. PTX, bis[indolylmaleimide I] (Bisindol), and PKC depletion indicate the presence of 100 ng/mL pertussis toxin, 1.0 μM Bisindol, and 1 μM PMA in the 16-h preincubation. Cell lysates were then prepared and MAPK activity assayed. Data shown are the fold increase of MAPK activity relative to control nonstimulated cells (mean ± SE, $n = 3$).

CHO cells. These data indicate that GalR2 mediates MAPK activation via a signaling pathway that employs a PTX-sensitive G protein and PKC.

Previous studies have demonstrated distinct PTX-sensitive signaling pathways leading to MAPK activation in CHO cells (*39*, *44*). One pathway employs the $\beta\gamma$-subunit of Gi and is independent of PKC. A separate pathway utilizes the α subunit of Go and is PKC-dependent. While GalR1 appears to mediate MAPK via the Gi signaling pathway, the profile of GalR2-mediated MAPK activation is consistent with the Go-mediated signaling pathway. To confirm that Gi$\beta\gamma$ mediates the stimulation of MAPK activity by GalR1 but not GalR2, the effect of βARKct expression on MAPK stimulation was determined. βARKct binds to the $\beta\gamma$-subunit of several G proteins and specifically inhibits G$\beta\gamma$-mediated signaling without disrupting Gα-mediated signaling (*30*). While galanin stimulated a 2−2.5-fold increase of MAPK activity in CHO cells transiently expressing GalR1 or GalR2, galanin-stimulated MAPK activity in CHO cells transiently expressing GalR1 was completely inhibited by expression of βARKct. In contrast, βARKct did not affect galanin-stimulated MAPK activity in CHO cells transiently expressing GalR2 (Figure 5). Activation of MAPK activity mediated by the M1 muscarinic receptor (M1AChR), which has been shown to utilize the α-subunit of Go in CHO cells (*39*), was also unaffected by βARKct expression (Figure 5). By contrast, LPA displayed βARKct-sensitive MAPK activation (Figure 5), as expected with Gi$\beta\gamma$ signal transduction.

The ability of GalR1 and GalR2 to activate signal transduction through Gq was examined by assessing the ability of galanin to stimulate inositol phosphate (IP)

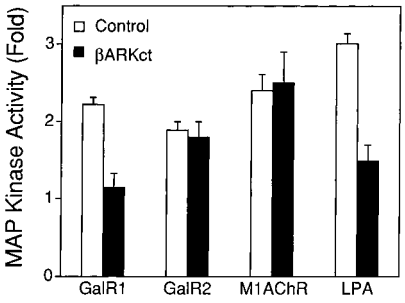

FIGURE 5: Effect of βARKct expression on GalR1- and GalR2-mediated MAPK stimulation. CHO cells were transiently cotransfected with plasmid DNA encoding GalR1, GalR2, or M1 AChR cDNA and vector alone (open bars) or plasmid containing the βARKct cDNA (filled bars). Transfected cells were incubated for 2 days and then serum-starved overnight. Cells were then stimulated with galanin for GalR1 and GalR2 activation, carbachol (1.0 mM) for M1ACh receptor activation, and LPA (10 mM) for LPA receptor activation for 5 min at 37 °C. Cell lysates were prepared and MAPK activity measured as described in Figure 4. Data show the fold increase of MAPK activity (mean ± SE, $n = 3$).

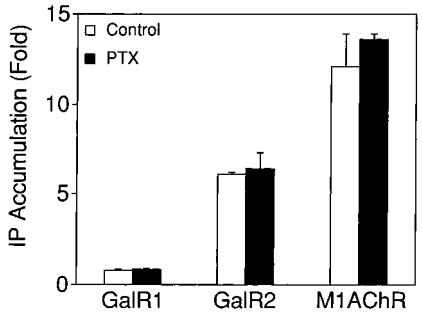

FIGURE 6: Inositol phosphate accumulation in CHO cells mediated by GalR1, GalR2, or M1AChR. CHO cells were transiently transfected with plasmid DNA encoding GalR1, GalR2 or M1AChR and incubated for 2 days. Cells were then preincubated for 24 h with myo[^{3}H]inositol. Cells were stimulated with 100 nM galanin or 1.0 mM carbachol for 45 min. IP accumulation was measured and expressed as fold increase of basal levels, which were defined as 1.0. Where indicated, pertussis toxin (100 ng/mL) was present during the prelabeling time period. Data indicate mean ± SE ($n = 3$).

production in CHO cells transiently expressing GalR1 or GalR2. Galanin was unable to stimulate IP production beyond basal levels in CHO cells expressing GalR1. In contrast, galanin stimulated a 6−7-fold increase in IP accumulation in CHO cells expressing GalR2 (Figure 6). CHO cells transiently expressing M1 muscarinic receptor responded to carbachol stimulation with a 13−14-fold increase in IP production (Figure 6). The increase in IP production mediated by GalR2 and M1AChR was insensitive to PTX pretreatment, indicating that this signaling is mediated via Gq rather than through a Gi/Go protein (only Gi/Go proteins are sensitive to PTX; Gs/Gq proteins are not). Similar results were observed in COS-7 cells transiently transfected with GalR1, GalR2, or M1AChR cDNA (data not shown). The results demonstrate that GalR2, but not GalR1, is capable of coupling to a PTX-insensitive, Gq signaling pathway.

DISCUSSION

The diversity of the physiological functions exerted by galanin suggests involvement of multiple galanin receptors activating distinct intracellular signaling pathways. We have

systematically studied the signal transduction pathways of the two recently cloned galanin receptors, GalR1 and GalR2. Our data reveal that, upon activation by galanin, the GalR1 and GalR2 receptors initiate strikingly different signaling pathways.

Several lines of evidence suggest that GalR1 is coupled to a Gi protein, and not to any other major G proteins. (1) The inhibition of forskolin-stimulated cAMP production mediated by GalR1 is PTX sensitive (*12*, *40*). (2) Similar to the LPA receptor, which mediates MAPK activation via Gi, the GalR1-mediated increase in MAPK activity is sensitive to PTX and to expression of the $\beta\gamma$-specific inhibitor βARKct (Figure 5). (3) GalR1 does not appear to be coupled to Gs since GalR1 stimulation does not cause an increase in cAMP accumulation (Figure 2). (4) GalR1-mediated MAPK activation is not dependent on PKC activity, which is required for Go-mediated MAPK activation. (5) GalR1 is not linked to Gq, as galanin does not activate IP production in GalR1/CHO cells (Figure 6) or in transfected COS cells.

Like GalR1, GalR2 does not mediate an increase in cAMP production, suggesting an inability to couple to Gs (Figure 2). However, in contrast to GalR1, the GalR2 galanin receptor appears capable of coupling to several other G proteins. GalR2 mediates a modest inhibition of cAMP production, demonstrating coupling to Gi (Figure 3). GalR2 also mediates MAPK activation via a PTX-sensitive, PKC-dependent mechanism that utilizes the α-subunit of a G protein, consistent with the MAPK signaling pathway mediated by Go (Figures 4 and 5). GalR2 is capable of mediating PTX-insensitive IP production in CHO and COS cells, demonstrating an ability to positively couple to Gq. The ability of GalR2 to couple to a wide range of G proteins is in marked contrast to GalR1, but it is consistent with other G protein-coupled receptors which are capable of multiple G protein activation. Similar to GalR2, M1 muscarinic receptor couples to Gq and Go in CHO cells (*39*). In addition, the α2-adrenergic receptor also couples to multiple G proteins within a single host cell type (*45*).

The actions of galanin may be regulated at several levels. Since different pharmacophores within the galanin peptide have differential affinities for different galanin receptor subtypes (*17*), it is possible that differential processing of galanin could lead to selective activation of particular receptor subtypes. In addition, galanin action appears to be regulated at the level of the receptor. Identification of multiple galanin receptor subtypes suggests the potential that these receptors may mediate distinct functions. The differential tissue distribution of the three cloned galanin receptors also suggests that different receptor subtypes may be responsible for specific galanin actions. The present study suggests that galanin actions may also be regulated by differential intracellular signaling mechanisms. Since the expression of GalR1 is detected at significant levels only in the brain and spinal cord, GalR1 may mediate some of the central functions through Gi-linked pathways. As GalR2 couples through at least three distinct G proteins, it seems likely that activation of GalR2 may lead to regulation of a wide range of both peripheral and central physiological functions.

In summary, we have systematically analyzed intracellular G protein-mediated signaling pathways, including Gs, Gi, Go, and Gq, that are capable of being stimulated in response to activation of the GalR1 and GalR2 galanin receptors. GalR1 appears to efficiently couple only to Gi, without activation of Gs, Go, or Gq. GalR2, in contrast, appears more permissive and can pleiotropically couple through three distinct G protein classes with an approximate rank order of Go = Gq > Gi (Table 1). The definition of these detailed signaling pathways will aid in understanding galanin-regulated function and may provide a vital connection between the previously described physiological actions of galanin and the more recent discoveries of multiple functional galanin peptides and galanin receptor subtypes.

Table 1: Differential Signaling of the GalR1 and GalR2 Galanin Receptors[a]

G protein		GalR1	GalR2	second messenger/effector
Gs		−	−	cAMP
Gi				
	α subunit	++	+	cAMP
	$\beta\gamma$ subunit	++	−	MAPK
Go		−	++	MAPK
Gq/G11		−	++	IP

[a] The coupling profiles of GalR1 and GalR2 to the four distinct G protein classes are summarized. Coupling to Gs was determined by measuring the effect of galanin on the basal levels of intracellular cAMP (Figure 2). Coupling to the α- and $\beta\gamma$-subunits of Gi was determined by measuring inhibition of forskolin-stimulated cAMP and galanin-evoked MAPK activity by galanin and galanin analogs (Figures 3 and 4). Go and Gq/11 were profiled by assaying PTX-insensitive MAPK activation and IP production, respectively (Figures 5 and 6). The symbols "++", "+", and "−" denote preferable, intermediate, and lack of coupling, respectively.

ACKNOWLEDGMENT

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. Bartfai, T. (1995) in *Psychopharmacology: The Fourth Generation of Progress* (Bloom, F. E., and Kupfer, D. J., Eds.) pp 563−571, Raven Press, New York.
3. Crawley, J. N. (1995) *Regul. Pept. 59*, 1−16.
4. Dunning, B. E., Ahren, B., Veith, R. C., Bottcher, G., Sundler, F., and Taborsky, G. J., Jr. (1986) *Am. J. Physiol. 251*, E127−E133.
5. Katsoulis, S., Clemens, A., Morys-Wortmann, C., Schworer, H., Schaube, H., Klomp, H. J., Folsch, U. R., and Schmidt, W. E. (1996) *Scand. J. Gastroenterol. 31*, 446−451.
6. Maggi, C. A., Patacchini, R., Santicioli, P., Giuliani, S., Turini, D., Barbanti, G., Giachetti, A., and Meli, A. (1990) *Naunyn Schmiedeberg's Arch. Pharmacol. 341*, 256−261.
7. Schepp, W., Prinz, C., Tatge, C., Hakanson, R., Schusdziarra, V., and Classen, M. (1990) *Am. J. Physiol. 258*, G596−G602.
8. Yagci, R. V., Alptekin, N., Rossowski, W. J., Brown, A., Coy, D. H., and Ertan, A. (1990) *Scand. J. Gastroenterol. 25*, 853−858.
9. Crawley, J. N., Austin, M. C., Fiske, S. M., Martin, B., Consolo, S., Berthold, M., Langel, U., Fisone, G., and Bartfai, T. (1990) *J. Neurosci. 10*, 3695−3700.
10. Xu, Y., Song, J., Bruno, J. F., and Berelowitz, M. (1993) *Biochem. Biophys. Res. Commun. 193*, 648−652.
11. Kaplan, L. M., Hooi, S. C., Abraczinkas, D. R., Strauss, M. R., Davidson, M. D., Hsu, D. W., and Koenig, J. I. (1991) in *Galanin: A new multifunctional peptide in the neuro-endocrine system* (Hokfelt, T., Bartfai, T., Jacobowitz, D., and Ottosom,

D., Eds.) Wenner-Gren International Symposium Series, Vol. 58, McMillan Press, London.
12. Parker, E. M., Izzarelli, D. G., Nowak, H. P., Mahle, C. D., Iben, L. G., Wang, J., and Goldstein, M. E. (1995) *Mol. Brain Res. 34*, 179−189.
13. Howard, A. D., Tan, C., Shiao, L.-L., Palyha, O. C., McKee, K. K., Weiger, D. H., Feighner, S. D., Cascieri, M. A., Smith, R. G., Van Der Ploeg, L. H. T., and Sullivan, K. A. (1997) *FEBS Lett. 405*, 285−290.
14. Wang, S., Hashemi, T., He, C., Strader, C., and Bayne, M. (1997) *Mol Pharmacol. 52*, 337−343.
15. Habert-Ortoli, E., Amiranoff, B., Loquet, I., Laburthe, M., and Mayaux, J. F. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9780−9783.
16. Wang, S., He, C., Maguire, M., Clemmons, A., Burrier, R., Guzzi, M., Strader, C., Parker, E., and Bayne, M. (1997) *FEBS Lett. 411*, 225−230.
17. Wang, S., He, C., Hashemi, T., and Bayne, M. (1997) *J. Biol. Chem. 272*, 31949−31952.
18. Federman, A. R., Conklin, B. R., Schrader, K. A., Reed, R. R., and Bourne, H. R. (1992) *Nature 356*, 159−161.
19. Lustig, K. D., Conklin, B. R., Herzmark, P., Taussig, R., and Bourne, H. R. (1993) *J. Biol. Chem. 268*, 13900−13905.
20. Winitz, S., Russell, M., Qian, N.-X., Gardner, A., Dwyer, L., and Johnson, G. L. (1993) *J. Biol. Chem. 268*, 19196−19199.
21. Krapivinsky, G., Krapivinsky, L., Wickman, K., and Clapman, D. E. (1995) *J. Biol. Chem. 270*, 29059−29062.
22. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E., and Clapham, D. E. (1987) *Nature 325*, 321−326.
23. Wickman, K., Iniguez-Lluhi, J., Davenport, P., Taussig, R. A., Krapivinsky, G. B., Linder, M. E., Gilman, A., and Clapham, D. E. (1994) *Nature 368*, 255−257.
24. Lopez-Ilasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S., and Wetzker, R. (1997) *Science 275*, 394−397.
25. Katz, A., Wu, D., and Simon, M. I. (1992) *Nature 360*, 686−688.
26. Blenis, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5889−5892.
27. van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L. M., and Lefkowitz, R. J. (1995) *Nature 376*, 781−784.
28. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Lefkowitz, R. J. (1996) *J. Biol. Chem. 271*, 12133−12136.
29. Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T., and Lefkowitz, R. J. (1996) *J. Biol. Chem. 271*, 19443−19450.
30. Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Lefkowitz, R. J. (1994) *J. Biol. Chem. 269*, 6193−6197.
31. Crespo, P., Xu, N., Simonds, W. F., and Gutkind, J. S. (1994) *Nature (London) 369*, 418−420.
32. Faure, M., Voyno-Yasenetskaya, T. A., and Bourne, H. R. (1994) *J. Biol. Chem. 269*, 7851−7854.
33. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L., and Moolenaar, W. H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1257−1261.
34. Howe, L. R., and Marshall, C. J. (1993) *J. Biol. Chem. 268*, 20717−20720.
35. de Vries Smits, A. M. M., Burgering, B. M. T., Leevers, S. J., Marshall, C. J., and Bos, J. L. (1992) *Nature (London) 357*, 602−604.
36. Leevers, S. J., Paterson, H. F., and Marshall, C. J. (1994) *Nature (London) 369*, 411−414.
37. Albas, J., van Corven, E. J., Hordijk, P. L., Milligan, G., and Moolenaar, W. H. (1993) *J. Biol. Chem. 268*, 22235−22238.
38. van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T., and Moolenaar, W. H. (1989) *Cell 59*, 45−54.
39. van Biesen, T., Hawes, B. E., Raymond, J. R., Luttrell, L. M., Koch, W. J., and Lefkowitz, R. J. (1996) *J. Biol. Chem. 271*, 1266−1269.
40. Smith, K. E., Forray, C., Walker, M. W., Jones, K. A., Tamm, J. A., Bard, J., Branchek, T. A., Linemeyer, D. L., and Gerald, C. (1997) *J. Biol. Chem. 272*, 24612−24616.
41. Cheng, Y., and Prusoff, W. H. (1973) *Biochem. Pharmacol. 22*, 3099−3108.
42. Samama, P., Cotecchia, S., Costa, T., and Lefkpwitz, R. J. (1993) *J. Biol. Chem. 268*, 4625−4636.
43. Moolenaar, W. H. (1995) *J. Biol. Chem. 270*, 12949−12952.
44. van Biesen, T., Luttrell, L. M., Hawes, B. E., and Lefkowitz, R. J. (1996) *Endocr. Rev. 17*, 698−714.
45. Raymond, J. R. (1995) *Am. J. Physiol. 269*, F141−F158.

BI9728405