Specificity of Metabotropic Glutamate Receptor 2 Coupling to G Proteins

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Received March 26, 2002; accepted October 8, 2002 This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Metabotropic glutamate receptor 2 (mGluR2) is a class 3 G protein-coupled receptor and an important mediator of synaptic activity in the central nervous system. Previous work demonstrated that mGluR2 couples to pertussis toxin (PTX)-sensitive G proteins. However, the specificity of mGluR2 coupling to individual members of the Ga family is not known. Using heterologously expressed mGluR2 in rat sympathetic neurons from the superior cervical ganglion (SCG), the mGluR2/G protein coupling profile was characterized by reconstituting coupling in PTX-treated cells expressing PTX-insensitive mutant Gα proteins and Gβγ. By employing this method, it was demonstrated that mGluR2 coupled strongly with Gaq, Gai11, Gai2, and Gai3, although coupling to Gai was less efficient. In addition, mGluR2 did not seem to couple to the most divergent member of the Gai family, Gai, although Gai2 coupled strongly to the endogenous α2 adrenergic receptor. To determine which Gα proteins may be natively expressed in SCG neurons, the presence of mRNA for various Gα proteins was tested using reverse transcription-polymerase chain reaction. Strong bands were detected for all members of the Gai family (Ga, Ga11, Gai2, Gai3, Gai) as well as for Gai1 and Gai2. A weak signal was detected for Gai and no Gai mRNA was detected.

Metabotropic glutamate receptors (mGluRs) are members of the class 3 G protein-coupled receptor family, which includes the calcium sensing receptor and the GABAB1 receptor, among others (Conn and Pin, 1997). There are eight known mammalian mGluR genes (mGluR1–8), which play diverse roles in the nervous system, including the modulation of synaptic transmission from both pre- and postsynaptic locations and regulation of synaptic plasticity. In addition, mGluRs also play a role in mediating sensory transduction (Bortolotto et al., 1994; Conn and Pin, 1997; Wilsch et al., 1998). mGluRs have been divided into three groups based on sequence homology, sensitivity to pharmacological agents, and G protein-coupling specificity (De Blasi et al., 2001). Group II (mGluRs 2 and 3) and group III mGluRs (mGluRs 4, 6–8) are known to couple exclusively to the pertussis toxin (PTX)-sensitive Gai family of G proteins (Tanabe et al., 1992, 1993; Saugstad et al., 1994), whereas group I mGluRs couple to multiple classes of G proteins (Abe et al., 1992; Aramori and Nakanishi, 1992; Pin et al., 1992; Joly et al., 1995).

The mechanism of mGluR/G protein coupling has been examined in several studies (Pin et al., 1995; Gomez et al., 1996; Blahos et al., 1998; Mary et al., 1998). Clearly, activation of G proteins by mGluRs in response to agonist binding involves regions of the receptor that are distinct from those of the class 1 G protein-coupled receptors. Coupling of mGluRs to G proteins seems to involve the proximal end of the intracellular C-terminal tail (Mary et al., 1998) and part of the second intracellular loop (Pin et al., 1995; Gomez et al., 1996). Chimeric group II/group I mGluRs in which these regions from a group I mGluR were inserted into mGluR3 were able to couple to phospholipase C (similar to wild-type group I mGluRs; Gomez et al., 1996). In addition, residues on the C-terminal tail of group I mGluRs seem to be involved in coupling to Gai, because this region has been shown to participate in phospholipase C activation (Mary et al., 1998). Thus, although many studies have examined the molecular basis of mGluR coupling to distinct G protein families (Gomez et al., 1996; Blahos et al., 1998), detailed studies of the G protein coupling specificity of an mGluR within a single G protein family have not been performed. Such studies may begin to shed light on the molecular basis for specificity in systems such as synaptic terminals in the central nervous system, where several types of G protein-coupled receptor are present.

PTX is a valuable tool for the study of heterotrimeric G proteins. By ADP-ribosylating the last cysteine residue in the extreme C terminus of Gai proteins (present only on G proteins in this family), PTX treatment selectively uncouples

ABBREVIATIONS: mGluR, metabotropic glutamate receptor; PTX, pertussis toxin; RT-PCR, reverse transcription-polymerase chain reaction; SCG, superior cervical ganglion.
these G proteins (Milligan, 1988). Consequently, mutation of this cysteine to another residue renders the resulting Ga PTX-insensitive. Therefore, when treating cells expressing a given G_{i/o}-coupled receptor with PTX to inactivate endogenous G_{i/o} proteins, the G protein-coupling specificity of the receptor to G proteins within this family can be examined by heterologously expressing individual Ga CG or CI mutants and examining coupling. This method has been used to examine the coupling of other G protein-coupled receptors (Taussig et al., 1992; Senogles, 1994; Wise et al., 1997; Jeong and Ikeda, 2000).

In this study, the G protein specificity of mGluR2 for Ga proteins in the G_{i/o} family was examined by reconstituting mGluR coupling in PTX-treated cells through expression of PTX-insensitive Ga (C-terminal Cys to Gly) mutants in sympathetic neurons from the rat superior cervical ganglion (SCG). In addition, to determine which subtypes of Ga proteins may be endogenously expressed in SCG neurons, the presence of mRNA for nine different Ga subunits was determined using RT-PCR.

Materials and Methods

Cell Isolation, DNA Injection, and Plasmids. A detailed description of the cell isolation and cDNA injection protocol is published elsewhere (Ikeda, 1997). The animal protocols used were approved by the Institutional Animal Care and Use Committee. Briefly, both SCGs were removed from adult Wistar rats (175–225 g) after decapitation, and incubated in Earle’s balanced salt solution (Invitrogen, Carlsbad, CA), 0.6 mg/ml collagenase D (Roche Applied Science, Indianapolis, IN), and 0.05 mg/ml DNase I (Sigma Chemical, St. Louis, MO) for 1 h at 35°C. Cells were then centrifuged (50 g), transferred to minimum essential medium (Fisher Scientific, Pittsburgh, PA), plated on poly(L-lysine)-coated 35-mm polystyrene dishes, and incubated overnight with PTX (0.5 ng/ml; List Biological, Campbell, CA) containing 0.45 mg/ml trypsin (Worthington Biochemicals, Freehold, NJ), 0.6 mg/ml collagenase D (Roche Applied Science, Indianapolis, IN), and 0.05 mg/ml DNase I (Sigma Chemical, St. Louis, MO) for 1 h at 37°C before DNA injection. After injection, cells were incubated overnight at 37°C and patch-clamp experiments were performed the following day. Where indicated, neurons were incubated overnight with PTX (0.5 ng/ml; List Biological, Campbell, CA) in the culture media.

Injection of cDNA was performed with an Eppendorf 5246 microinjector and 5171 micromanipulator (Madison, WI) 4 to 6 h after cell isolation. Plasmids were stored at -20°C as a 1 µg µl stock solution in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8). Rat mGluR2 was injected at 50 ng/µl (pC; Promega, Madison, WI). Construction of the PTX-insensitive mutants of Ga_{i1} and Ga_{o1} has been described previously (Jeong and Ikeda, 2000). For reconstitution experiments, all Ga cDNAs (pC; Promega) were injected at 5 to 6 ng/µl with bovine Gβ1 and Gγ2 (from M. I. Simon, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA) injected at 10 ng/µl each (pC; Promega). Neurons were coinfected with “enhanced” green fluorescent protein cDNA (0.005 µg/µl; pEGFP-N1; BD Clontech Laboratories) to facilitate later identification of successfully injected cells. All inserts were sequenced using an automated DNA sequencer (ABI 310; Applied Biosystems, Foster City, CA). PCR products were purified with QIAGEN (Valencia, CA) silica membrane spin columns before restriction digestion and ligation. Plasmids were propagated in XL1-blue bacteria (Stratagene, La Jolla, CA) and midipreps prepared using QIAGEN anion exchange columns.

Electrophysiology and Data Analysis. Patch pipettes were made from 7052 glass (Garin Glass, Claremont, CA) and had resistances of 1 to 4 MΩ. Series resistances were 2 to 6 MΩ before electronic compensation, which was typically ≥80%. Ruptured patch whole-cell recordings were made with an Axopatch 200A patch-clamp amplifier (Axon Instruments, Union City, CA). Voltage protocol generation and data acquisition were performed using custom software on a Macintosh Quadra series computer (Apple Computer, Cupertino, CA) with a MacADIOS II data acquisition board (G.W. Instruments, Somerville, MA). Currents were low-pass-filtered at 5 kHz using the four-pole Bessel filter in the patch-clamp amplifier, digitized at 2 to 5 kHz and stored on the computer for later analysis. Experiments were performed at 21 to 24°C (room temperature). Data analysis was performed using Igor software (Wavemetrics, Lake Oswego, OR).

The external (bath) solution contained 155 mM Tris, 20 mM HEPES, 10 mM glucose, 10 mM CaCl$_2$, and 0.0003 mM tetrodotoxin, adjusted to pH 7.4 with methanesulfonic acid; osmolality, 320 mOsm/kg. The internal (pipette) solution contained: 120 mM N-methyl-D-glucamine, 20 mM tetaethylammonium methanesulfonic acid, 11 mM EGTA, 10 mM HEPES, 10 mM sucrose, 1 mM CaCl$_2$, 4 mM MgATP, 0.3 mM Na$_2$GTP, and 14 mM Tris-creatine phosphate, pH 7.2; osmolality, 300 mOsm/kg. PTX was obtained from List Biological (Campbell, CA) and applied to cells overnight at 500 ng/ml as indicated. L-Glutamate (100 µM) was used as the agonist for mGluR2. All drugs and control solutions were applied to cells using a custom gravity-driven perfusion system, positionned ~100 µm from the cell, that allowed rapid solution exchange (∼250 ms). The degree of mGluR-mediated calcium current inhibition (and norepinephrine-mediated inhibition, where indicated) was calculated as the maximal inhibition of the current in the presence of drug compared with the last current measurement before application of the drug.

RT-PCR. To test for the presence of mRNA coding for each of the nine Ga subunits (i1–i3, o, q, n, 1, and 15), unique 18–25-base primer pairs from coding or 3' noncoding sequences were identified using MacVector software (Accelrys Inc., Princeton, NJ). Potential primers were constrained by length, GC content, melting temperature, and product size (see Table 1 for primer list and expected product sizes). Where possible, longer PCR products that were more likely to span introns were selected to reduce the contribution of genomic DNA. In addition, samples were treated with DNase as part of the RNA isolation procedure. Each potential primer was then BLAST-searched to rule out the presence of homologous or identical sequences.

### TABLE 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Expected Product Size</th>
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<tr>
<td>Ga6</td>
<td>nt</td>
</tr>
<tr>
<td>5'-CTGTGGAATGGATGGACAAAGAGAG-3'</td>
<td>300</td>
</tr>
<tr>
<td>5'-AAAGGTAATGGGGTCTTCAGATG-3'</td>
<td>482</td>
</tr>
<tr>
<td>5'-CTGGACTATGCCATCGTTCGAG-3'</td>
<td>210</td>
</tr>
<tr>
<td>5'-ACACCTAACCTCCTGTGTCGGGG-3'</td>
<td>453</td>
</tr>
<tr>
<td>5'-CAAGATGTTTGTATGTTTGGTGGTC-3'</td>
<td>250</td>
</tr>
<tr>
<td>5'-AGGATGAGGAGGTCGTCAGA-3'</td>
<td>432</td>
</tr>
<tr>
<td>5'-TGATGATGGAGGTGTCTGTGA-3'</td>
<td>531</td>
</tr>
<tr>
<td>5'-GGTTGATGTTGAGGATGCTCAGG-3'</td>
<td>147</td>
</tr>
<tr>
<td>5'-TCTCAGGACGATGTTAGCCAGTGTC-3'</td>
<td>300</td>
</tr>
<tr>
<td>5'-AGGATGCTGCTTGGTGGAAG-3'</td>
<td>432</td>
</tr>
</tbody>
</table>
sequences present in other known rat mRNAs. The primer sets for each Gsu as well as the size (number of bases) of the expected product are shown in Table 1. RT-PCR was performed using the QIAGEN One-Step RT-PCR kit on 40 ng of isolated total RNA (annealing temperature of 60–63°C, for 30–35 cycles) from dissociated rat SCG cultures using the QIAGEN Nanoeasy total RNA isolation kit. As a positive control, primers were constructed to detect the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) message (5′- CACAAAGGTCATCATCTCCG-3′, and 5′- AGA-CAACCTGTCCTCAGTGACG-3′, producing an expected product of 501 bases). Negative controls were performed with the GAPDH primers in the absence of RNA. Primers were obtained from Operon Technologies (Alameda, CA). RT-PCR products were run on 3% agarose precast gels from Bio-Rad (Hercules, CA).

**Western Blotting.** Homogenates (10%, w/v) were made from combined SCGs dissected from an adult rat, and protein concentrations were determined using the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Polyacrylamide gels (10%) were used for protein fractionation and parallel gels were stained with Coomasie blue to verify loading of proteins, separation, and sample integrity. Proteins were then transferred to polyvinyl difluoride membranes for immunodetection. The membranes were blocked for ≥1 h with 5% powdered milk in 25 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, then probed with anti-Gα antibodies at dilutions in the same medium. Antibodies used were anti-Gα4 (1:200, 4°C overnight; Santa Cruz Biotechnology, Santa Cruz, CA) and Calbiochem (San Diego, CA) and anti-Gα1 (1:5,000, 1 h, 22°C; PerkinElmer Life Sciences, Boston, MA), anti-Gαq/11 (1:10,000, 1 h, room temperature; Calbiochem), polyclonal anti-Gαq/11 (Calbiochem) and monoclonal anti-Gαq/11 (both at 1:1000, 4°C overnight; LabVision, Fremont, CA). Horseradish peroxidase-conjugated donkey anti-rabbit (Cell Signaling Technology, Beverly, MA) was used (1:1000) for detection. Peroxidase activity was detected with SuperSignal West Pico (Pierce Biotechnology) and visualized using a Kodak Image Station 1000 or with X-ray film (XAR-2; Eastman Kodak, Rochester, NY).

**Results**

**Calcium Current Inhibition by Heterologously-Expressed mGluR2.** Neurons isolated from the rat SCG do not functionally express mGluRs (Ikeda et al., 1995). HeterologOUS expression of distinct subtypes of mGluRs in SCG neurons is therefore a useful model system for studying the properties of individual mGluR subtypes. Cells expressing mGluR2 after intranuclear cDNA injection respond to application of 100 μM L-glutamate with a fast and potent inhibition of the predominantly N-type (Zhu and Ikeda, 1994) whole-cell calcium current (Ikeda et al., 1995; Kammermeier et al., 2000). The time course of this inhibition is illustrated in Fig. 1A (see inset for samples of control and Glu-inhibited current traces). The ‘triple-pulse’ voltage protocol (Elmslie et al., 1990) was used to illustrate the voltage-dependent nature of the modulation and to measure basal facilitation as an indicator of free Gβγ levels (see below). The average magnitude of mGluR2-mediated calcium current inhibition was 59 ± 2% (n = 31; Fig. 1C). Cells expressing mGluR2 and treated overnight with 500 ng/ml PTX did not exhibit any detectable calcium current inhibition in response to Glu application (Fig. 1B). Calcium current inhibition in PTX-treated cells was 2 ± 0.3% (n = 24; Fig. 1C). This result confirms previous observations that mGluR2 couples to PTX-sensitive, Gαq, G proteins (Chavis et al., 1994; Ikeda et al., 1995).

**Reconstitution of G Protein Coupling after PTX Treatment.** The strategy for examining the specificity of mGluR2 coupling to Gαq proteins is illustrated in Fig. 2A. First, cells were intranuclearly injected with cDNA for mGluR2 plus Gβ1, Gγ2 (this Gβγ combination was chosen for its ability to robustly modulate N-type calcium currents when expressed in SCG neurons), and a PTX-insensitive mutant (or naturally PTX-insensitive wild-type) Ga. Next, cells were treated overnight with PTX to inactivate endogenous Gαq proteins. Finally, calcium current facilitation was examined to determine the Gαq/Gβγ stoichiometry.

The calcium current modulatory pathway used by mGluR2 here is Gβγ-mediated and voltage-dependent (Herlitze et al., 1996; Ikeda, 1996). This is evident from the slowed activation kinetics of the inhibited currents and from the ‘facilitation’ observed after a strong depolarizing prepulse (Bean, 1989; Elmslie et al., 1990) (Fig. 1A, inset). These features are hallmarks of the Gβγ-mediated calcium current inhibitory pathway. Commonly, facilitation is defined as the current in the postpulse divided by the current at the same time in the prepulse (the first test pulse to +10 mV). Thus, facilitation can be used as a quantitative measure of relative free Gβγ levels in the cell. Overexpression of Gβγ alone mimics this modulation and produces basal currents with slow activation and strong basal facilitation (ikeda, 1996; Garcia et al., 1998).
1998; Ruiz-Velasco and Ikeda, 2000). Overexpression of Ga subunits alone produces basal currents with facilitation < 1, because of strong buffering of endogenously expressed Gβγ. Under these conditions, agonist-induced Gβγ-mediated calcium current modulation is occluded.

As illustrated in Fig. 2B, heterologous expression of Ga and Gβγ resulted in cells with currents that were placed into three functional categories. In the first category were placed all cells that exhibited strong basal facilitation (>1.3, chosen arbitrarily because basal facilitation this high was rare in control cells in this study: 1 of 55 cells). This level of facilitation was an indication of excess free Gβγ. The second category included those cells with basal facilitation < 1, indicating excess Ga, resulting from Gβγ buffering by expressed Ga. The third category included cells with basal facilitation between 1 and 1.3, indicating a good functional stoichiometric balance of Ga and Gβγ. Therefore, Ga/Gβγ-expressing cells with basal facilitation in this range were chosen for analysis (Jeong and Ikeda, 2000). In addition to determining stoichiometric balance, these criteria provide a control for expression levels of different Ga subunits, assuming that levels of Gβαγ remain relatively constant.

**mGluR2 Coupling to Gαo, G Proteins.** After treatment with PTX, cells expressing mGluR2 exhibited no detectable calcium current inhibition in response to 100 μM L-glutamate (Glu; as described in Fig. 1B). Over this background, PTX-insensitive Gaα proteins [with a Cys-to-Gly mutation in the extreme C terminus, denoted GaC351G (or GaαC352G)] were expressed with Gβγ to reconstitute coupling and examine the specificity of mGluR2/G protein interactions. Calcium current inhibition in PTX-treated cells expressing GaαoC351G (and Gβαγ) was strong (Fig. 3A, a), indicating that GluR2 couples efficiently to Gaαo. The magnitude of calcium current inhibition in these cells was indistinguishable from paired control cells (recorded the same days; Fig. 3B, □). Calcium currents in

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**Fig. 2. Strategy for PTX-insensitive Ga reconstitution experiments.** A, graphic depiction of the reconstitution procedure. First, cells were injected intranuclearly with cDNA for mGluR2, Gaα CG, Gβγ cDNA. 2. Treat with PTX to inactivate native Gaαo proteins. 3. Examine Ica facilitation to determine Ga/Gβγ stoichiometry.

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**Fig. 3. Reconstitution of mGluR2 coupling with GaαoC351G, GaαoC351G, and Gaα. A, time course and sample currents (a) of calcium current inhibition in a PTX-treated SCG neuron expressing mGluR2, Gaα CG, Gβγ, and Gαo. Scale bars in a denote 1 nA and 20 ms. B, Dose dependence of the calcium current inhibitory response for glutamate of wild-type mGluR2-expressing cells (●) and cells expressing mGluR2 reconstituted with PTX-insensitive GaαoC351G (□). Each data set was fit to the equation Bmax[Glut]/(EC50 + [Glut]), with Bmax fixed at 1. Data were normalized to inhibition at 1 μM within each cell. Fits revealed EC50 values for the control and reconstituted conditions of 1.3 and 3.8 μM, respectively. B, bar graph illustrating average (+ S.E.M.) calcium current inhibition in PTX-untreated controls (Control), PTX-treated controls (PTX), both mGluR2-expressing), and Gaα reconstituted (PTX/Gaα CG) Gβγ, PTX-treated, expressing mGluR2, GaCG - or wild type Gaα, Gβα, and Gγ2, for Gaαo (□), Gaαo CG (□), Gaαo (□), and Gaα (□). Number of cells is indicated in parentheses.
PTX-treated cells reconstituted with \( \alpha_{ob} \)C351G were inhibited 66 ± 5 % \((n = 5)\) upon Glu application. Calcium currents in paired control cells (PTX-untreated) were inhibited 68 ± 4 % \((n = 6)\), whereas currents in paired PTX-treated control cells were inhibited 0.5 ± 0.4 % \((n = 6)\). Dose-response curves for calcium current inhibition in control and \( \alpha_{ob} \)C351G-reconstituted cells are illustrated in Fig. 3A, b. EC\(_{50}\) values for the two groups, determined by fitting to a single-site binding isotherm equation (see Fig. 3 legend), were comparable. Control cells had an EC\(_{50}\) of 1.3 μM, compared with 3.8 μM for \( \alpha_{ob} \)C351G-reconstituted cells. These data provide evidence that the CG mutation in the C terminus of the Ga subunit did not detectably alter the receptor-G protein interaction.

Reconstitution with \( \alpha_{om} \)C351G was less efficient, exhibiting calcium current inhibition of only 27 ± 10 % \((n = 7)\) compared with 54 ± 5 % \((n = 6)\) in control cells from the same experimental days (Fig. 3B). PTX-treated control cells from the same preparations were inhibited 2 ± 1 % \((n = 6)\). These data indicate that mGluR2 couples more efficiently to \( \alpha_{ob} \)C351G than to \( \alpha_{om} \)C351G, a surprising result because the extreme C terminus, a region demonstrated to be critical in receptor/G protein interaction (Hamm et al., 1988; Conklin et al., 1993), is nearly identical in these two splice variants. Poor expression of the \( \alpha_{om} \)C351G construct cannot sufficiently explain these results because Ga expression levels were balanced with \( \beta \gamma \) expression. Finally, mGluR2 seemed to be unable to couple to \( \alpha_{om} \). Calcium current inhibition in PTX-treated cells reconstituted with \( \alpha_{om} \) (a naturally PTX-insensitive member of the \( \alpha_{om} \) family) was virtually undetectable at only 3 ± 2 % \((n = 6)\), compared with 61 ± 4 % \((n = 3)\) in PTX-untreated cells, and 1 ± 0.6 % \((n = 3)\) in PTX-treated control cells (Fig. 3B).

As negative controls, similar reconstitution experiments were performed using wild-type \( \alpha_{om} \) or wild-type \( \alpha_{ob} \) (Fig. 4). As expected, no detectable calcium current inhibition was evident in PTX-treated cells expressing either \( \alpha_{om} \), which is not coupled to mGluR2, or \( \alpha_{ob} \), the PTX-sensitive wild-type Ga. Stoichiometrically balanced PTX-treated cells coexpressing mGluR2, \( \beta_{1} \gamma_{2} \), and \( \alpha_{q} \) were not inhibited by Glu (0 ± 0.3 %, \( n = 5 \)), compared with inhibitions of 61 ± 4 % \((n = 3)\) in PTX-untreated paired control cells and 1 ± 0.6 % \((n = 3)\) in PTX-treated paired control cells. Similarly treated cells coexpressing mGluR2, \( \beta_{1} \gamma_{2} \), and wild-type \( \alpha_{q} \) were inhibited −1 ± 2 % \((n = 2)\) by Glu, compared with 47 ± 12 % \((n = 3)\) and 3 ± 0.2 % \((n = 3)\) in PTX-untreated and -treated control cells, respectively.

As a positive control for expression, \( \alpha_{om} \) was coexpressed with \( \beta_{1} \gamma_{2} \) to reconstitute coupling to the natively expressed \( \alpha_{2} \) adrenergic receptor in PTX-treated cells. Coupling of the \( \alpha_{2} \) adrenergic receptor to \( \alpha_{om} \) in SCG neurons has been demonstrated previously (Jeong and Ikeda, 1998). In PTX-treated cells coexpressing \( \beta \gamma \) and \( \alpha_{om} \) (in functional stoichiometric balance), 10 μM norepinephrine (NE) inhibited calcium currents 69 ± 3 % \((n = 3)\). Paired PTX-untreated and -treated cells were inhibited 74 ± 1 % \((n = 3)\) and 13 ± 5 % \((n = 3)\), respectively. These data demonstrate that \( \alpha_{om} \) is expressed and is capable of coupling a G protein-coupled receptor to calcium channels in SCG neurons.

Finally, the remaining members of the \( \alpha_{om} \) family were examined. Figure 5A, inset, illustrates the time course and sample currents from a PTX-treated, mGluR2-expressing cell reconstituted with \( \beta \gamma \) and \( \alpha_{om} \)C352G. Glu-mediated calcium current inhibition in this cell was potent, indicating that mGluR2 couples efficiently to \( \alpha_{om} \) in this system. On average, PTX-treated cells whose mGluR2 coupling was reconstituted with \( \alpha_{om} \)C352G were inhibited 48 ± 10 % \((n = 8)\).
by Glu, compared with 53 ± 6% (n = 5) and 2 ± 1% (n = 3) in paired PTX-un-treated and -treated control cells, respectively (Fig. 5B). In addition, mGlur2 seemed to be similarly capable of coupling to Gα11 and Gαq (Fig. 5B). Reconstitution using the CG mutants of these Gα proteins also exhibited efficient coupling to calcium currents. PTX-treated cells coexpressing mGlur2, Gα11C352G, and Gβγ2 were inhibited 57 ± 9% (n = 5) by Glu. Inhibition in PTX-un-treated, paired control cells was 68 ± 2% (n = 4) and in PTX-treated, paired control cells inhibition was 2 ± 2% (n = 3). PTX-treated cells coexpressing mGlur2, GαqC352G, and Gβγ2 were inhibited 68 ± 1% (n = 4) by Glu. Inhibition in PTX-un-treated, paired control cells was 51 ± 7% (n = 4), and in PTX-treated, paired control cells, inhibition was 3 ± 1% (n = 3).

Endogenous Expression of Gα Proteins in SCG Neurons. To determine which Gα proteins may be expressed natively in SCG neurons, and to shed some light on possible receptor/G protein interactions of natively-expressed receptor/G protein pairs (or in the case of mGlur2, heterologously expressed receptor/native G protein pairs), RT-PCR was used to detect mRNA for several Gα proteins. Unique primer sets were designed for several Gα proteins, including o, i1–3, z, q, 11, s, and 15. In addition, primers for the housekeeping gene GAPDH were used as a positive control. Table 1 lists the primer sequences and predicted product size for each Gα primer set. Figure 6A shows the results of RT-PCR reactions targeting each of the Gαo and Gαq proteins. In each case, a clear band at the predicted product size was detected. In addition, the GAPDH positive and negative (no RNA) controls are shown. These data suggest that SCG neurons may potentially express each member of the Gαo and Gαq protein family.

In addition to the Gαo and Gαq proteins, the presence of message for several other Gα proteins was tested using RT-PCR. As Fig. 6B indicates, bands were detected at the predicted product sizes for Gαi1, Gαi11, and Gαs. However, the Gαi3 band seemed much weaker than that of the other Gα proteins. This suggests that the Gαi3 mRNA is unstable or perhaps present at lower levels than the other Gα proteins tested, but poor hybridization by the selected primers is the more likely cause of the weak signal. Therefore, one can only infer that Gαi3 message is present in rat SCGs. In addition, previous studies have confirmed the presence of Gαi3 in SCG neurons, as well as Gαi1 and Gαi11 (Haley et al., 1998). Finally, Gα15 message was undetectable in RNA from SCG neurons. This result was expected because Gα15 expression is confined to hematopoietic cells (Wilkie et al., 1991).

Western blotting was used to confirm the results of RT-PCR experiments where specific antibodies were available, as judged by detection of recombinant proteins (Fig. 6C). Of the antibodies tested, only three (anti-Gαi1, anti-Gαq11, and anti-Gαs) seemed specific as judged by detection of recombinant proteins (see Fig. 6C). Others, namely Gαi1, Gαi11, and Gαs, detected protein from SCG near the appropriate molecular weight, but also detected at least one inappropriate recombinant control (data not shown), so the presence of specific proteins could not be determined with confidence. The positive result for Gαs RNA was surprising. Previously, Gαs protein has been shown to be present in brain and few other tissues at low levels (Fong et al., 1988; Matsuoka et al., 1988; Casey et al., 1990) and, to our knowledge, has not been demonstrated in sympathetic neurons. However, when Western blots were performed, Gαs could not be detected with protein from either SCG or hippocampus using any of three commercially available antibodies (see Materials and Methods). Thus, the RT-PCR experiment suggesting the presence of Gαs in SCG neurons could be neither confirmed nor refuted with Western blotting experiments.

Discussion

The aim of this study was to characterize the G protein coupling profile of mGlur2, a Gαo-coupled receptor. This was achieved by treating mGlur2-expressing cells with PTX to inactivate endogenous Gαo proteins and coexpressing various PTX-insensitive mutants of Gα proteins with Gβγ to reconstitute coupling, measured as the degree of calcium current modulation upon Glu application. In addition, RT-PCR was used to detect messenger RNA coding for various Gα proteins endogenously expressed in SCG neurons. The RT-PCR results were confirmed with Western blots where possible.

Although mGlur2 is known to couple exclusively to the Gαo family of G proteins, a comprehensive characterization of coupling with members within this family has not been reported. Such data may shed light on a potential source of specificity, particularly in systems in which several G protein-coupled receptors are known to coexist. For example, the existence of a subset of Gα proteins in a nerve terminal coupled with the knowledge of G protein coupling capabilities of the expressed receptors could lead to a more complete understanding of the specific roles of individual receptors. To date, tools are unavailable to distinguish between the individual members of every G protein family, but there is some evidence that Gα proteins may be selectively localized in nerve terminals. In the large calyx preparation of the chick ciliary ganglion, several Gα proteins have been shown to express in the synaptic terminals and associate with the active site, whereas other Gα proteins (namely Gαz and Gαo), known to express elsewhere in the cells, are excluded from close association with the membrane at release sites (Mirotznik et al., 2000). If similar selective localization of Gα proteins within a family also occurs, then this in combination with unique receptor/G protein coupling profiles may underlie the specific physiological roles of the receptors.

The data presented here demonstrate that mGlur2 can couple efficiently to Gαi10, Gαi11, Gαi12, and Gαi13, and less efficiently to Gαs. Additionally, mGlur2 does not seem to couple to the more divergent member of the Gαo family, Gαo. (Fong et al., 1988; Matsuoka et al., 1988). Particularly intriguing is the finding of selectivity between Gαs and Gαo. This is surprising because there are few amino acid changes between these two splice variants and only one in the extreme C terminus (N/K at −10 from the C-terminal end of the
mouse sequence used for expression in this study; Fig. 6C). The extreme C terminus is believed to play an important role in receptor/G protein interaction and is generally thought to be critical for selectivity in receptor interactions (Hamm et al., 1988; Conklin et al., 1993). In fact, several studies have shown that receptor selectivity can be conveyed to Gα chimeras by swapping only the most distal five amino acids (Conklin et al., 1993; Gomeza et al., 1996; Blahos et al., 1998). Therefore, the finding that mGluR2 coupling is selective for Gαob over Gαoa suggests that other regions of Gα may also be important in coupling to receptors, at least to class 3 G protein-coupled receptors such as mGluRs. The small number of amino acid changes across these variants (of which just 15 are nonconservative changes) could provide a useful starting point for investigation into the molecular basis for mGluR/G protein interaction.

Although the measured signal (calcium current modulation) is Gβγ-mediated, the identity of βγ released from the various Gα subunits can be ruled out as the source of observed differences in signal strength because the same Gβγ subunits (Gβ1,γ2) were used in each experiment in this study. Although these subunits have been shown to produce robust voltage dependent calcium current modulation, specificity of signaling does not seem to come from specific Gβγ subunit combinations (Ruiz-Velasco and Ikeda, 2000). In addition, the two Gα subunits that displayed inefficient coupling with mGluR2 in this study (Gαa and Gαoa) have been demonstrated to couple strongly to endogenous receptors in this system by a previous study from this laboratory (Jeong and Ikeda, 2000), using the same Gαoa,G5151 and Gαo constructs that were used in the present study. Finally, the differences in coupling specificity between the heterologously expressed mGluR2 in this study and the endogenous α2 adrenergic receptor may lead to speculation that differences in coupling result from differential access to molecular scaffolds. However, because distinct coupling profiles have been reported for various endogenously expressed receptors (Jeong and Ikeda, 2000), this explanation is unlikely to account for all of the observed differences in G protein coupling across receptor types. Changes in mGluR2 expression levels might have also influenced coupling. However, this did not seem to be the case here. Results from each group were consistent despite the variability in expression levels that normally results from cDNA injection as judged by GFP expression.

A tacit assumption of these studies is that the C-to-G mutation in Gα does not greatly influence receptor-G protein coupling fidelity. However, the mutated residue lies within a region of Gα identified as a critical determinant of receptor/G protein coupling (Hamm et al., 1988; Conklin et al., 1993). Thus, the mutation may influence G protein coupling to mGluRs. Although we cannot completely rule out this possibility, studies of PTX-resistant Gα subunits indicate that although the efficacy of partial agonists is altered, general characteristics of coupling are maintained (Bahia et al., 1998). Moreover, in the current study, neither the EC50 nor the maximal effect of reconstituted Gαab, was significantly altered from control (Fig. 3). Thus, the strategy is clearly useful for determining G protein coupling profiles within the context of the required mutation. However, extrapolation of these data to native proteins requires some caution and alternative approaches will be required to definitively establish a coupling profile.

Results from this study confirm the conclusions from some recent studies. Gomeza et al. (1996) and Blahos et al. (1998) demonstrated that Gα chimeras containing the N terminus of Gαi and the extreme C terminus of either Gαo or Gαc could couple to phospholipase C via mGluR2, but similar Gαi/Gαc chimeras could not. These data indicate that mGluR2 is capable of coupling to variants of Gαo and Gαc, but not to Gαa, as was demonstrated here. It should be noted, however, that the experiments in the Gomeza et al. (1996) and Blahos et al. (1998) papers were unable to distinguish coupling to Gαoa from that of Gαab, or among Gαi1, Gαi2, and Gαi3. Also, the assay for coupling in those studies was dependent on PLC activation by chimeric receptors. Therefore, only differences in Gα coupling resulting from sequence variations in the C-terminal 5 amino acids could be detected. It should also be noted that under the conditions described here, mGluR2 seemed unable to couple to similar Gαi/Gαc chimeras as described in the above studies (not shown).

One recent study examined coupling of several endogenously expressed receptors in cultured hippocampal neurons using a strategy similar to that described here (Straiker et al., 2002). Although mGluR2 was not examined, a previously expressed group III mGluR was tested and seemed unable to couple to the PTX-insensitive Gα proteins tested (Gαoa, Gα11-3). However, the authors note that the initial signal (synaptic inhibition by a group III mGluR agonist) was small, which may have contributed to a difficulty in reconstitution.

The RT-PCR results described above demonstrate the presence of mRNA from rat SCG for Gαo1, Gαi1, Gαi2, Gαi3, and Gαa. In addition, mRNA coding for Gαoa, Gαi1, and Gαa was detected, although the Gαoa signal seemed weaker than the other Gα subunits tested. These data are interesting considering the findings regarding G protein coupling specificity of heterologously expressed mGluR2. For example, although Gαa seems to be expressed strongly in SCG neurons, it is likely that any coupling between mGluR2 and Gαa is primarily via Gαab, because mGluR2/Gαa coupling seems inefficient. In addition, although Gαo may be present in SCG neurons, it does not seem to contribute to calcium current modulation via heterologously expressed mGluR2 in this system. Regarding the naturally expressed α2 adrenergic receptor, previous work has demonstrated strong coupling to Gαoa, Gαab, Gαi2, and Gαc, but weak coupling to Gαi1 and Gαi3 (Jeong and Ikeda, 2000). This is particularly interesting in light of the finding that all members of the Gα family seem to be expressed in SCGs. The implication of these findings is that in native neuronal systems, signal specificity may arise, at least in part, from selective coupling to individual members within G protein families. It should be noted, however, that although care was taken to minimize the number of glial cells in the SCG preparation from which the RNA was isolated, it is likely that some were present and may have contributed to results. Therefore, future studies should be performed using RNA isolated from single SCG neurons to confirm the results presented here.

The presence of Gαo mRNA from SCG neurons was unexpected. Previously, Gαo protein has been shown to be present in brain and some other tissues at low levels (Fong et al., 1988; Matsuoka et al., 1988; Casey et al., 1990), but not in sympathetic neurons. Here, we show that Gαo message is present in sympathetic neurons from the rat SCG, but we were unable to confirm (or refute) this result by demonstrat-
ing the presence of the Goα protein with Western blotting. Western blots were also performed to confirm the presence of other Gα proteins. However, because of the lack of specificity of most anti-Gα antibodies tested (as judged by recognition of various recombinant Gα proteins), many of these experiments produced less than meaningful results. Exceptions were Goα2 (which was detected in SCG and did not recognize even the closely related Goα1, or Goαq) Goαq (although this antibody did not distinguish recombinant Goα1, and Goαq (Fig. 6).

In summary, the G-protein-coupling profile of mGluR2 was characterized using heterologous expression in SCG neurons treated with PTX and reconstituting coupling to calcium currents by coexpressing PTX-insensitive Goαq, proteins with Gβγ. mGluR2 was found to couple strongly to Goαi, Goαi2, and Goαi3, and less strongly to Goαo. No coupling with Goαq was observed. Finally, several Gα mRNAs were detected in rat SCG with RT-PCR, including Goαo, Goαi1–3, Goαq, and Goαs. Finally, message for Goα15 was absent, as expected, because of its unique expression in hematopoietic tissue (Wilkie et al., 1991).

Acknowledgments

We thank M. King for valuable technical assistance.

References


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