Coexpression of Full-Length γ-Aminobutyric AcidB (GABA_B) Receptors with Truncated Receptors and Metabotropic Glutamate Receptor 4 Supports the GABA_B Heterodimer as the Functional Receptor

RICHARD SULLIVAN, ANNE CHATEAUNEUF, NATHALIE COULOMBE, LEE F. KOLAKOWSKI JR., MICHAEL P. JOHNSON, TERENCE E. HEBERT, NATHALIE ETHIER, MICHEL BELLEY, KATHLEEN METTERS, MARK ABRAMOVITZ, GARY P. O’NEILL, and GORDON Y. K. NG

Departments of Biochemistry, Molecular Biology and Chemistry, Merck Frosst Center for Therapeutic Research, Kirkland, Quebec, Canada (R.S., A.C., N.C., M.B., K.M., M.A., G.P.O., G.Y.K.N.); Departments of Pharmacology and Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas (L.F.K., M.P.J.); and Institut de Cardiologie de Montreal, Research Center, Montreal Heart Institute, Montreal, Quebec, Canada (T.E.H., N.E.)

Accepted for publication January 27, 2000

ABSTRACT

Direct evidence is lacking to show whether the γ-aminobutyric acid (GABA)B gb1-gb2 heterodimer is the signaling form of the receptor. In this study, we tested whether gb1a or gb2 subunits when coexpressed with truncated receptors or metabotropic glutamate receptor mGluR4 could form functional GABA receptors. Coexpression of the ligand binding N-terminal domain of gb1a or the C-terminal portion of gb1a composing the seven-transmembrane segments and intracellular loops with gb2 could not reconstitute functional receptors. We next examined whether mGluR4, which forms homodimers and is structurally related to GABA_B, could act as a surrogate coreceptor for gb1 or gb2. The coexpression of mGluR4 and gb1a led to the expression of gb1a monomers on cell surface membranes as determined by immunoblot analysis and flow cytometry. However, mGluR4-gb1a heterodimers were not formed, and membrane-expressed gb1a monomers were not functionally coupled to adenylyl cyclase in human embryonic kidney 293 cells or activated inwardly rectifying potassium (Kir) channels in Xenopus oocytes. Similarly, the coexpression of mGluR4 and gb2 led to nonfunctional GABA receptors. GABA-activated distal signaling events resulted only after the coexpression and heterodimerization of gb1 and gb2. Taken together with the truncated receptor studies, the data suggest that a high degree of structural specificity is required to form the functional GABA_B receptor that is a gb1-gb2 heterodimer.

γ-Aminobutyric acid (GABA) is the most widely distributed inhibitory amino acid neurotransmitter in the vertebrate central nervous system. GABA activities are mediated by three types of GABA receptors, which are classified according to biochemical and pharmacological criteria into ionotropic GABAA/GABAC receptors and metabotropic GABA_B receptors (see Mody et al., 1994, for a review).

Received for publication November 12, 1999.

1 The work in the laboratory of T.E.H. was supported by the Medical Research Council of Canada, the Heart and Stroke Foundation of Canada, and the Fonds de la Recherche en Santé du Québec.

ABBREVIATIONS: GABA, γ-aminobutyric acid; mGluR, metabotropic glutamate receptor; GPCR, G protein-coupled receptor; TM, transmembrane domain; Kir, inwardly rectifying potassium channel; HEK, human embryonic kidney; PCR, polymerase chain reaction; bp, base pair(s).
responsible for ligand binding (Kaupmann et al., 1998b). GABA$_B$ receptors are structurally related to metabotropic glutamate receptors (mGluRs) and together with calcium-sensing receptors belong to the family 3 (C) G protein-coupled receptors (GPCRs).

Unlike other GPCRs, recombinant gb1 and gb2 receptors are functionally inactive when expressed individually (Jones et al., 1998; White et al., 1998; Ng et al., 1999b). It is generally accepted that the functional GABA$_B$ receptor results from the coexpression and translocation of the gb1 subunit to the cell surface by the gb2 subunit as a heterodimer (Jones et al., 1998; White et al., 1998; Kaupmann et al., 1998a; Kuner et al., 1999; Ng et al., 1999b). Yeast two-hybrid screening showed that a coiled-coil motif in the carboxyl tails of gb1 and gb2 receptors likely mediate gb1-gb2 heterodimerization (White et al., 1998; Kuner et al., 1999). However, several studies have reported that gb1 (Kaupmann et al., 1997, 1998b) and gb2 (Kuner et al., 1999, Martin et al., 1999) expressed alone can activate inwardly rectifying potassium channel (Kir) channels and/or inhibit cAMP production. To clarify the structural requirements necessary for the expression of the functional GABA$_B$ receptor, we expressed full-length and truncated gb1a and gb2 receptors, alone and together, and examined their ability to form functional receptors. To further test the specificity of GABA$_B$ receptor heterodimerization, we also examined whether coexpression of gb1a or gb2 with the structurally related mGluR4 receptor was sufficient to form a functional GABA receptor. Our data show that 1) coexpression of gb1a and gb2 leads to GABA-mediated activation of K$^+$ currents and inhibition of cAMP production, 2) coexpression of truncated gb1a receptors with gb2 does not reconstitute functional GABA receptors, 3) coexpression of gb1a and mGluR4 leads to expression of gb1a monomers on the cell surface, 4) coexpression of gb1a and mGluR4 does not result in mGluR4-gb1a heterodimers, and 5) coexpression of gb1a or gb2 with mGluR4 does not result in functional GABA receptors. We conclude that the gb1-gb2 heterodimer is the functional GABA$_B$ receptor species.

**Materials and Methods**

**Receptor Expression Constructs.** The recombinant murine gb1a (herein referred to as gb1a) receptor (GenBank accession number AF114168) exhibits 98.5% amino acid identity to the human gb1a receptor (GenBank accession number AJ225028) and was used as a model for gb1a receptors. The gb1a cDNA was constructed from two expressed sequence tags (IMAGE Consortium clone identification numbers 472408 and 319196). The partial cDNAs were assembled by polymerase chain reaction (PCR) using the following oligonucleotides: 472408 sense, 5'-GG GAATTC TGG ATC TCC GTC TCA GTT CTC TCC CCT-3'; 472408 antisense, 5'-GG GAATTC TGG ATA TAA CGA GGC TGG TGG TAG ATG TTA AA-3'; 319196 sense, 5'-CCA GAATTC CCA GCC CAA CCT GAA CAA TC-3'; and 319196 antisense, 5'-CG GCGGCGGC TCA CTT GTA AAG CAA ATG TA-3', which amplified two fragments corresponding to the 5' 2100 bp and 3' 1000 bp of the gb1a receptor cDNA coding region. PCR products were cloned into the TA-Cloning vector pCRll-TOPO vector (Invitrogen, San Diego, CA) according to the manufacturer's directions. The EcoRI fragment from PCR cloning using 472408 primers and the EcoRI/NcoI product from PCR cloning using 319196 primers were ligated as a 2903-bp open reading frame into pcDNA3.1 (Invitrogen) vector (Stratagene, La Jolla, CA) or pcDNA3.1 (Invitrogen) vector (Stratagene).

**Receptor Expression in Whole Cells.** Receptor DNAs (6 ¿ total DNA/1.2 × 10$^6$ cells) were transiently transfected into COS-7 (American Type Culture Collection, Rockville, MD) or HEK 293 (Aurora Bioscience) cells using 3 ¿ of Lipofectamine reagent (Life Technologies, Ontario, Canada) according to the manufacturer's instructions. Transient gb1 and gb2 coexpression studies were performed using 1:1 ratio of receptor DNAs.

Stable gb2 receptor-expressing cells were selected by growth in puromycin (5 ¿/ml) containing medium and limiting dilution. The gb2 receptor RNA expression levels in clones were determined by dot-blot analysis. Briefly, RNA was prepared using TRIzol reagent (Life Technologies, Madison, WI) using pcDNA2.1 plasmids containing the gb1a, N-gb1a, or C-gb1a DNAs. Translation products were analyzed by electrophoresis on 8 to 16% Tris-glycine SDS gradient gels (Novex). Hybridization was performed using a $^{32}$P-labeled DNA probe from the cDNA sequence of the N-terminal fragment of the gb1a receptor (gb1a), composed of an initiating methionine and amino acid positions 588 to 942, was generated by PCR using primer pairs CFP-CJ7845 (5'-ACC ACT GCT AGC ACC GCC ATG CTA CCT CGT CTT CCT CTC G-3') and CRP-CJ7846 (5'-CAG CTC ATG TAA ACG AAA TGT CCA GCC GGC CGG CGT CAC CA-5'). Similarly, the C-terminal fragment of the gb1a receptor (C-gb1a), composed of an initiating methionine and amino acid positions from amino acid position 1 to 625, was generated by PCR. The coding sequence of the C-terminal fragment of the gb1a receptor was amplified by use of primer pairs NFP-CJ7843F139 (5'-ACC ACT GCT AGC ACC GCC ATG CTG CTT CGT CCT TCT CTC C-3') and NBP-CJ7848R0G GTG CGA GCC ATA TAG GTT TTA AGG GTC GGC GCC CGC CCT GAC CA-5').

**In Vitro Receptor Expression.** In vitro coupled transcription/translation reactions were performed in the presence of $[^{35}$S)methionine in the TNT Coupled Reticulocyte Lysate system (Promega, Madison, WI) using pcDNA2.1 plasmids containing the gb1a, N-gb1a, or C-gb1a DNAs. Translation products were analyzed by electrophoresis on 8 to 16% Tris-glycine SDS gradient gels (Novex). The gb2 receptor-expressing cells were selected by growth in puromycin (5 ¿/ml) containing medium and limiting dilution. The gb2 receptor RNA expression levels in clones were determined by dot-blot analysis. Briefly, RNA was prepared using TRIZOL reagent (Life Technologies, Ontario, Canada) according to the manufacturer's instructions. Transient gb1 and gb2 coexpression studies were performed using 1:1 ratio of receptor DNAs.

**Membranes and Immunoprecipitation.** Cells were washed twice with cold PBS, collected by centrifugation at 100g for 7 min, and resuspended in 10 ml of Buffer A (5 mM Tris-Cl, 2 mM EDTA containing 1 ¿ protease inhibitor cocktail Complete tablets (Boehringer-Mannheim, Indianapolis, IN), pH 7.4, at 4°C). Cells were disrupted by Polytron homogenization and centrifuged at 100g for 7 min to pellet unbroken cells and nuclei, and the supernatant (S1) was collected. The S1 supernatant was centrifuged at 40,000g for 20 min to recover the crude membrane (P2) fraction. Membranes were then washed once with Buffer A, centrifuged (27,000g for 20 min) and resuspended in Buffer A, and stored at -80°C. The supernatant (S1) was centrifuged at 100,000g for 30 min to recover total cellular membranes that were washed and stored in Buffer A. Pro-
tein content was determined using the Bio-Rad Protein Assay Kit (Ontario, Canada) according to the manufacturer’s instructions.

For the immunoprecipitation experiments, 100,000g membranes were solubilized with digitonin, and samples were immunoprecipitated with a mouse anti-FLAG M2 (Kodak IBI, New Haven, CT) or anti-ε-myc 9E10 antibody (Santa Cruz Biochemicals, Santa Cruz, CA) affinity resin essentially as previously described (Ng et al., 1994). Immunoprecipitates were washed and submitted to immunoblot analysis as described later.

**Immunoblot Analysis.** Crude membranes (40,000g) were solubilized in SDS sample buffer consisting of 50 mM Tris-HCl, pH 6.5, 10% SDS, 10% glycerol, and 0.003% bromophenol blue with 10% 2-mercaptoethanol and separated on 8 to 16% Tris-glycine SDS gradient gels. The full-length gβ1 receptor and N-gβ1a truncated receptor were detected using affinity-purified rabbit polyclonal antibodies 1713.1 raised against the peptide-acetyl-DVNSRRIDL-PDYELKL-amide and antibody 1713.2 raised against the peptide-acetyl-CLTHNPTRVKLPEK-amide in the N-terminal tail of the gβ1 receptor. gβ2 receptors were detected using affinity-purified rabbit polyclonal antibody 1630.1 raised against the peptide-acetyl-CSGKTPQQYEREYNK-amide and antibody 1630.2 raised against the peptide-acetyl-QDVQRFSVNRDLTC-amide of the gβ2 receptor. The characterization of gβ1 and gβ2 antibodies have been reported elsewhere (Belley et al., 1999; Ng et al., 1999b). Specific immunoreactivity was revealed by secondary antibody coupled to horseradish peroxidase and chemiluminescence detection using the Renaissance Western Blot Chemiluminescence Reagent Plus kit (New England Nuclear, Boston, MA). The whole-cell expression of the C-gβ1a truncated receptor was detected using a GABA<sub>δ</sub> receptor antibody AB1531 (Chemicon Int, Ontario, Canada) raised against the peptide-acetyl-PSEPPDRLSCDGVHLY-amide in the C-terminal tail of the gβ1 receptor. Specific C-gβ1a immunoreactivity was revealed by a secondary antibody coupled to alkaline phosphatase and detected using the Immuno-Blot Alkaline Phosphatase Assay Kit (Bio-Rad).

**Densitometry.** Determinations of immunoreactive band intensity were made by scanning on a GS-719 calibrated imaging densitometer (Bio-Rad) and analyzed using ImageQuant 5.0 software (Molecular Devices, Sunnyvale, CA). In the immunoblot shown (see Fig. 3), a rectangular region was defined around the ~130-kDa band in the gβ1a/mGlur4 and gβ1a/FLAG28-bands and the corresponding region in gβ1 and pcDNA3.1 lanes. The pixel/density of the defined region was determined, and the background, as defined by pcDNA3.1, was subtracted from all subsequent band determinations. To ensure analysis in the linear range, X-ray films were exposed to immunoblots of 25- to 50-kDa proteins for various times.

**Receptor Binding Assays.** The synthesis of the [H<sup>125</sup>l]CGP17872 ([H<sup>125</sup>l]T3-(1R,3R)-3-[(4-azido-2-hydroxy-5-iodobenzoylamino)pentyl]hydroxphosphoryl)-2-(8-hydroxypropylamino)ethylbenzoic acid) photoaffinity label and conditions for receptor binding have been described elsewhere (Belley et al., 1999).

**Functional Assays.** cAMP determinations were made using a scintillation proximity assay kit (Amersham, Ontario, Canada). Briefly, HEK 293 cells were washed and detached, and 77,000 to 100,000 cells/well were resuspended in Hanks' balanced salt solution containing 25 mM HEPES, pH 7.4, 100 μM 4-(3-butoxy-4-methoxy-benzyl)-2-imadazolidinone (Ro 20-1724; BIOMOL Research Laboratories, Plymouth Meeting, PA) and incubated for 20 min at 37°C. Then, 2 μM forskolin and ligands (10<sup>-9</sup> to 10<sup>-3</sup> M) were added and incubated for 30 min at 37°C. Cells were lysed by boiling, and cAMP levels were determined by scintillation proximity assay according to the manufacturer’s instructions.

**GABA Receptors.** The characterization of gb1 and gb2 antibodies have been reported elsewhere (Belley et al., 1999; Ng et al., 1999b). Specific immunoreactivity was revealed by secondary antibody coupled to horseradish peroxidase and chemiluminescence detection using the Renaissance Western Blot Chemiluminescence Reagent Plus kit (New England Nuclear, Boston, MA). The whole-cell expression of the C-gb1a truncated receptor was detected using a GABA<sub>δ</sub> receptor antibody AB1531 (Chemicon Int, Ontario, Canada) raised against the peptide-acetyl-PSEPPDRLSCDGVHLY-amide in the C-terminal tail of the gβ1 receptor. Specific C-gb1a immunoreactivity was revealed by a secondary antibody coupled to alkaline phosphatase and detected using the Immuno-Blot Alkaline Phosphatase Assay Kit (Bio-Rad).

**Flow Cytometry.** Analysis was performed using live intact cells, which were incubated with primary antibodies for 1 h in Hanks' balanced salt solution, followed by incubation with secondary antibody conjugates under similar conditions. Goat anti-rabbit antibodies coupled with Alexa-488 (Molecular Probes, OR) were used to detect rabbit gb1 or gb2 antibodies. We analyzed 10,000 cells per condition with a Becton Dickinson (San Jose, CA) FACS Vantage SE flow cytometer configured to detect fluorescein isothiocyanate fluorescence.

**Results**

**Coexpression of Ligand Binding N-Terminal Domain of gβ1a or Transmembrane Domain (TM) 1 to 7 Segments of gβ1a with gb2 Does Not Result in Functional GABA Receptors.** We asked whether coexpression of N-terminal (N-gβ1a) and C-terminal (C-gβ1a) truncated gβ1 receptors with gb2 was sufficient to form functional GABA<sub>δ</sub> receptors. N-gβ1a composed the signal peptide and the entire extracellular N-terminal domain, including the putative TM 1 segment, which was retained to anchor and orient the protein in the plasma membrane (Fig. 1A). The C-gβ1a composed the receptor from TM 1 to 7 through to the C-terminal tail containing coiled-coil and PDZ (PSD-95, Disc-large, and ZO-1) domains for protein-protein interactions (Fig. 1A).

In vitro transcription/translation studies revealed the expression of a ~63-kDa N-gβ1a monomer and a ~40-kDa C-gβ1a monomer corresponding to the nonglycosylated forms of these receptors. In crude membranes prepared from whole cells, immunoblotting and [H<sup>125</sup>l]CGP17872 photoaffinity labeling revealed the expression of a ligand-binding ~95-kDa N-gβ1a species, presumably representing a glycosylated form of the receptor, and a mature nonglycosylated ~40-kDa C-gβ1a species that did not bind ligand (Fig. 1, B–D). Competition studies at N-gβ1a revealed a rank order of affinities of CGP17872 > SKF-97541 [3-aminopropyl(methyl)phosphonic acid] ≥ GABA ≥ (+)-baclofen > saclofen similar to gβ1a (data not shown), suggesting that N-gβ1a retains the pharmacological characteristics of the full-length receptor. The soluble N terminus of gβ1a alone was reported previously to be sufficient to specify agonist and antagonist binding, although agonist affinities were higher possibly because this construct lacked the TM 1 segment present in N-gβ1a, which may influence agonist affinities (Malitschek et al., 1999).

The ability of gβ1a, N-gβ1a, and C-gβ1a to form functional receptors with gb2 was determined in a stable high-level
Expression and photoaffinity labeling of gb1a and N- and C-terminal gb1a receptors. A, schematic representation of the gb1a receptor and the N-gb1a and C-gb1a receptor fragments showing the extracellular N-terminal domain and putative ligand binding lobes (L1–L2), TM segments, carboxyl-tail coiled-coil (CC), and PDZ interacting modules. B, cell-free expression of the pcDNA3.1 vector, gb1a, N-gb1a, and C-gb1a receptor constructs. The autoradiogram shown is from a 4-h exposure and is representative of two independent experiments. C, expression of ~130-kDa immunoreactive gb1a and ~95-kDa N-gb1a receptors as determined by immunoblotting with N-terminal gb1 antibodies 1713.1–1713.2. The ~40-kDa C-gb1a receptor fragment was determined by immunoblotting with C-terminal gb1 antibodies, gb1a, N-gb1a, and C-gb1a immunoreactive species are absent in pcDNA3.1-transfected COS-7 cells. The full-length gb1a monomer, N-gb1a, and C-gb1a receptor fragments are delimited in brackets. For each immunoblot condition, 50 μg of membrane protein was used. The immunoblot shown is representative of two experiments. D, autoradiograms showing [125I]CGP71872 photolabeling of the full-length ~130-kDa gb1a receptor (1-day exposure), ~95-kDa N-terminal gb1a fragment (12-day exposure), but not the ~40-kDa C-terminal gb1a receptor fragment (12-day exposure), in the absence (+) and presence (-) of 1 μM CGP71872. Labeling conditions were 50 and 100 μg/ml membrane protein for gb1a and truncated receptors, respectively. The autoradiograms shown are representative of three independent experiments.

Fig. 1. Expression and photoaffinity labeling of gb1a and N- and C-terminal gb1a receptors. A, schematic representation of the gb1a receptor and the N-gb1a and C-gb1a receptor fragments showing the extracellular N-terminal domain and putative ligand binding lobes (L1–L2), TM segments, carboxyl-tail coiled-coil (CC), and PDZ interacting modules. B, cell-free expression of the pcDNA3.1 vector, gb1a, N-gb1a, and C-gb1a receptor constructs. The autoradiogram shown is from a 4-h exposure and is representative of two independent experiments. C, expression of ~130-kDa immunoreactive gb1a and ~95-kDa N-gb1a receptors as determined by immunoblotting with N-terminal gb1 antibodies 1713.1–1713.2. The ~40-kDa C-gb1a receptor fragment was determined by immunoblotting with C-terminal gb1 antibodies, gb1a, N-gb1a, and C-gb1a immunoreactive species are absent in pcDNA3.1-transfected COS-7 cells. The full-length gb1a monomer, N-gb1a, and C-gb1a receptor fragments are delimited in brackets. For each immunoblot condition, 50 μg of membrane protein was used. The immunoblot shown is representative of two experiments. D, autoradiograms showing [125I]CGP71872 photolabeling of the full-length ~130-kDa gb1a receptor (1-day exposure), ~95-kDa N-terminal gb1a fragment (12-day exposure), but not the ~40-kDa C-terminal gb1a receptor fragment (12-day exposure), in the absence (+) and presence (-) of 1 μM CGP71872. Labeling conditions were 50 and 100 μg/ml membrane protein for gb1a and truncated receptors, respectively. The autoradiograms shown are representative of three independent experiments.

Fig. 2. GABA<sub>B</sub> receptor modulation of forskolin-stimulated cAMP synthesis in HEK 293 cells. A, gb2 hybridization signal from RNA dot-blot analysis (2-day exposure) of vector-transfected HEK 293 cells or gb2-expressing HEK 293 clones (gb2-46). Three percent of gb2-45 and 60% of gb2-46 cells (10,000 sampled) showed surface anti-gb2 FITC fluorescence over background by flow cytometry. B, gb1a constructs were transiently transfected into gb2 stable cell lines to examine the effect of receptor coexpression on modulation of cAMP synthesis. In this experiment, GABA mediated a dose-dependent inhibition of 2 μM forskolin-stimulated cAMP levels up to 60% with an IC<sub>50</sub> value of 49 nM in gb1-gb2 coexpressing cells (C). Expression of gb1a alone in HEK 293 (D) or coexpression of N-gb1a (E) or C-gb1a (F) with gb2 did not result in a GABA-mediated response. gb2-expressing cells (G) and vector-transfected HEK 293 cells (H) also exhibited no GABA activity. In this experiment, basal cAMP was 1.1 pmol/10<sup>6</sup> cells, which increased to 9.2 pmol/10<sup>6</sup> cells after 2 μM forskolin stimulation. In each reaction conducted in triplicate, 10<sup>6</sup> cells were used. Data are presented as the percentage of total cAMP synthesized in the presence of 2 μM forskolin alone. This experiment was replicated four times.

GB2-expressing HEK 293 cell line selected on mRNA and surface expression (Fig. 2A). As expected, GABA mediated a dose-dependent decrease (46–61%) in forskolin-stimulated cAMP levels (IC<sub>50</sub> = 49–321 nM) in stable gb2 HEK 293 cell lines transfected with gb1a but not when receptors were expressed alone (n = 4; Fig. 2B), indicating that gb1a and gb2 monomers are not functionally coupled to adenylyl cyclase in HEK 293 cells. GABA-mediated inhibition of cAMP synthesis was not observed after the coexpression of the ligand binding N-gb1a construct or C-gb1a in the clonal gb2 cell line (n = 4; Fig. 2B), indicating that the functional GABA<sub>B</sub> receptor requires both full-length gb1a and gb2 subunits for signaling via adenylyl cyclase.

Coexpression of mGluR4 Promotes Plasma Membrane Expression of gb1a but Not Function. mGluR4 can undergo dimerization and exhibits motifs required for protein-protein interactions. Thus, we reasoned that mGluR4 might act as a surrogate coreceptor for gb1a translocation and the functional expression of gb1a. We examined the ability of mGluR4 to promote the plasma membrane expression of gb1a by immunoblot analysis and flow cytometry and the ability to form heterodimers with gb1a by differential immunoprecipitation and blotting.

Densitometric analysis of immunoblots of crude (40,000g) membranes prepared from COS-7 cells coexpressing gb1a and FLAG-gb2 showed a ~15-fold increase in the expression of a ~130-kDa gb1a over the staining in gb1a-expressing cells (Fig. 3A). White et al. (1998) showed that the coexpression of gb1a and gb2 resulted in the membrane expression of a ~130-kDa mature glycosylated gb1a monomer. c-myc-mGluR4 promoted a smaller 4- to 7-fold increase in the membrane expression of a ~130-kDa gb1a monomer identical in size to the gb1a monomer in cells coexpressing gb1a
Immunodetection of gb1a expressed individually or coexpressed with gb2 receptor-expressing cells. gb1a receptor antibodies 1713.1–1713.2 showed expression of gb1a monomers in crude membranes, although slightly less efficient than gb2 (Fig. 3A).

Because gb2 undergoes heterodimerization with gb1a for cell surface targeting, we asked whether mGluR4 can form heterodimers with gb1a. We used a differential coimmunoprecipitation and immunoblotting strategy similar to the one used to show gb1-gb2 heterodimers (Ng et al., 1999b). The gb1 receptor antibodies were used to blot gb1a immunoprecipitated with FLAG antibodies directed against gb2. Consistent with previous findings, the gb1a-gb2 heterodimer and gb1a monomer were only detected in gb1a/FLAG-gb2-coexpressing cells (Fig. 4C). The gb1 receptor antibodies were then used to blot gb1a immunoprecipitated with c-myc antibodies directed against mGluR4. In contrast, gb1a immunoreactivity was not detected in immunoprecipitate prepared from cells coexpressing gb1a and c-myc-mGluR4 (Fig. 4C), even though a ∼110-kDa immunoreactive species, likely corresponding to the glycosylated mGluR4 monomer (Han and Hampson, 1999), was detected in this sample with a mGluR4 antibody (Shigemoto et al., 1997; data not shown). This demonstrates that gb1a did not coimmunoprecipitate with c-myc-mGluR4. The gb1a immunoreactivity was not detected in c-myc antibody-immunoprecipitated samples from vector-transfected cells, FLAG-gb2-expressing cells, or gb1a receptor-expressing cells indicating that gb1a forms a specific heterodimer with gb2. Although GABA_B receptors undergo heterodimerization and mGluRs undergo homodimerization, these data are the first to demonstrate the structural specificity between these family C GPCRs.

To examine whether the mGluR4-promoted expression of the mature gb1a monomer is sufficient to result in functional GABA receptors, we coexpressed mGluR4 and gb1a in X. laevis oocytes and melanophores. Consistent with previous findings, coexpression of gb1a and FLAG-gb2 with Kir 3.1/3.2 in X. laevis oocytes resulted in a significant stimulation of Kir current in response to 100 μM GABA (297 ± 30.5% increase over control current, n = 11) measured at −80 mV, whereas modulation of Kir 3.1/3.2 was not seen in oocytes expressing gb1a or FLAG-gb2 individually (n = 11; Fig. 4A). GABA (100 μM) could not stimulate Kir current in oocytes coexpressing gb1a and mGluR4 (n = 11; Fig. 4A).

In melanophores transiently cotransfected with the gb1a and FLAG-gb2 receptors, GABA mediated a dose-dependent pigment aggregation response with an IC_{50} value of 0.6 to 8 μM (n = 4; Fig. 4B). GABA activity was not detected, testing concentrations up to 1 mM, in melanophores transiently cotransfected with c-myc-mGluR4 and gb1a or c-myc-mGluR4 and FLAG-gb2. Thus, gb1a receptors do not form functional GABA receptors after coexpression with mGluR4. The functional GABA receptor results only from the coexpression of gb1 and gb2 subunits.
Functional GABA<sub>B</sub> Heterodimers

**Discussion**

Native GABA<sub>B</sub> receptors are well known to couple to membrane K<sup>+</sup> channels as well as to adenylyl cyclase in neurons (Bowery and Enna, 2000). Therefore, we evaluated the ability of recombinant gb1a and gb2 receptors to modulate Kir channel activity in *X. laevis* oocytes and to inhibit cAMP levels in *X. laevis* melanophores and HEK 293 cells. Under our assay conditions, gb1a and gb2 receptors, when expressed alone, are nonfunctional, consistent with the intracellular retention of gb1 in the absence of gb2 (Couve et al., 1998; White et al., 1998), low agonist affinities of gb1 monomers (Kaupmann et al., 1997, 1998b), and the lack of detectable binding sites on gb2 (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999a). These results are discrepant with studies by Kaupmann et al. (1997), who reported that the rat gb1a receptor, when expressed alone, can inhibit forskolin-stimulated cAMP levels and that human gb1a and gb1b receptors can activate Kir channels in HEK 293 cells (Kaupmann et al., 1998b). In the latter study, however, modulation was only detectable in ~10% of the cells where current was measured and was significantly attenuated compared with other GPCRs. We have not detected any modulation by gb1a or gb2 alone for Kir 3.1/3.2, Kir 3.1/3.4, Kir 3.2, or Kir 3.4 (data not shown). Two studies have also reported that the gb2 receptor, when expressed alone, can mediate baclofen-inhibited forskolin-stimulated cAMP production (Kuner et al., 1999; Martin et al., 1999). As proposed by Martin et al. (1999), the discrepancy may be due to higher levels of stable gb2 receptor expression in their CHO cells achieved using inducible systems. Our clonal gb2 receptor-expressing HEK 293 cell line showed high RNA and surface gb2 receptor expression, but receptors were nonligand binding (data not shown) and exhibited no functional activity following up to 1 mM GABA or 1 mM (R)-baclofen treatment (data not shown). It will be important to determine the gb2 receptor density conferring functional activity, gb2 may bind a yet-to-be-discovered ligand. gb1 and gb2 monomer activity may also be cell line-dependent. A number of studies have highlighted the importance of an appropriate cell background in the identification of orphan receptors, in particular in the identification of CGRP and adrenomedullin receptors (McLatchie et al., 1998). It is possible that the functional expression of gb1 or gb2 receptors in certain cell lines is due to the coexpression of an endogenous surrogate coreceptor.

We reasoned that if the gb1-gb2 heterodimer were the active form of the GABA<sub>B</sub> receptor, this would be a specific functional interaction. We coexpressed a truncated N-terminal portion of gb1a, containing the major determinants for ligand binding, with gb2, and a C-terminal portion of gb1a, containing the TM 1 to 7 segments, extracellular and intracellular loops and carboxyl tail, with gb2. GABA did not mediate inhibition of forskolin-stimulated cAMP production in gb2-expressing cells transfected with N-gb1a, suggesting that although the ligand binding N termini of gb1a and gb2 are present, the C-terminal portion of gb2, absent the C-terminal portion of gb1a, is not sufficient alone to promote coupling to effector pathways. Although we determined that N-gb1a exhibits binding characteristics similar to gb1a whereas gb2 does not bind ligands, we did not determine whether high-affinity agonist binding result in cells coexpressing N-gb1a and gb2. The lack of high-affinity binding
likely does not explain the lack of function because GABA concentrations were tested up to 1 mM. The data suggest that N-gb1a and gb2 monomers and/or N-gb1a-gb2 heterodimer expressed in these cells are functionally inactive. GABA (up to 1 mM) also did not mediate inhibition of forskolin-stimulated cAMP production in gb2-expressing cells transfected with C-gb1a. This suggests that although C-gb1a contains the intracellular domains for G protein interactions and the coiled-coil domain for heterodimerization with gb2, the extracellular N terminus of gb2 is not sufficient in the absence of the N terminus of gb1a to bind and mediate the intrinsic activity of agonist. The C-gb1a and gb2 monomers and/or C-gb1a-gb2 heterodimers that are expressed in these cells are functionally inactive. The functional GABAB receptor coupled to the inhibition of adenyl cyclase with a nanomolar potency for GABA results only from the coexpression of the full-length gb1a and gb2 receptors. Thus, the most likely explanation is that the functional GABAB receptor is a pre-existing gb1-gb2 heterodimer with the major site for ligand binding and effector coupling conferred by gb1a.

The truncated receptor studies, however, do not rule out the possibility that once gb1 is expressed on the plasma membrane with gb2, the mature gb1 monomer is rendered functional. Membrane-expressed gb1 monomers may occur under certain cellular environments and could account for the reported ability of gb1 monomers to couple to adenyl cyclase in HEK 293 cells or Kir channels in X. laevis oocytes (Kaumann et al., 1997, 1998b). To address this, we asked whether mGluR4 could act as a surrogate coreceptor (translocator protein) for gb1a. The mGluR4 receptor shares many structural features with GABAB receptors, including protein-protein interacting PDZ and SCR domains (Kaumann et al., 1998b), and forms functional homodimers (Han and Hampson, 1999), making this a candidate coreceptor. It should be noted that mGluR4 does not exhibit a coiled-coil domain present in gb1 and gb2 that mediates the heterodimerization of these receptors (White et al., 1998; Kuner et al., 1999).

Coexpression of gb1a with gb2 resulted in the membrane expression of a mature gb1a monomer corresponding to the glycosylated form of the receptor (White et al., 1998). Coexpression of gb1a with mGluR4 also resulted in the membrane expression of a mature gb1a monomer, but mGluR4 was slightly less efficient than gb2 and did not form heterodimers. mGluR trafficking has been reported to involve an interaction with Homer/Vesl family of proteins (Ciruela et al., 1999; Roche et al., 1999), but a Homer/Vesl consensus sequence in gb1a is lacking. Possibly, mGluR4 transiently stabilizes gb1a in the endoplasmic reticulum such that it can fold/mature and traffic to the cell surface, but the mechanism remains unknown at this time. This, however, provided a model system to test whether mature gb1a monomers, in the absence of gb2, are functionally coupled. The coexpression of mGluR4 and gb1a in oocytes and melanophores did not result in the formation of active GABA receptors. Similar results were obtained after the coexpression of mGluR4 and gb2. This indicates that the functional GABAB receptor results only from the coexpression of gb1 and gb2 and that the functional receptor is a gb1-gb2 heterodimer.

The mode of gb1-gb2 heterodimer ligand binding and activation may resemble the insulin tyrosine kinase receptors that exist as preformed dimers that, on ligand binding, undergo transition to an active conformation (Weiss and Schlessinger, 1998). Future experiments planned using fluorescence donor-gb1 and fluorescence acceptor-gb2 pairs in fluorescence resonance energy transfer-based assays will be valuable in confirming the conclusions of this study. Of interest, a growing number of GPCRs have been reported to exist as dimers (Hebert and Bouvier, 1998), but the therapeutic importance is largely unclear. In the case of the dopamine D2 receptor, monomers and homodimers are differentially bound by butyrophenone and benzamide neuroleptic antagonists, which exhibit different side effects profiles (Ng et al., 1996). Coexpressed α- and β- opioid receptors result in a new receptor with distinct pharmacology (Jordan and Devi, 1999). gb1a, gb1b, and gb1c isoforms differ in their ligand-binding extracellular N-terminal domains. gb1a and gb1b differ in their extrasynaptic localizations (Billinton et al., 1999; Fritschy et al., 1999) but are colocalized with gb2 (Benke et al., 1999), raising the possibility that coexpression of gb1 isoforms with gb2 could result in pharmacologically and functionally distinct GABAB heterodimers.

Acknowledgments

We thank KevinClark for the preparation of manuscript figures, Ken MacDonald for technical assistance, and Louise Charlton for administrative assistance.

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Send reprint requests to: Dr. Gordon Y. K. Ng, Departments of Biochemistry, Molecular Biology and Chemistry, Merck Frosst Center for Therapeutic Research, 16711 TransCanada Hwy., Kirkland, Quebec, H9H 3L1 Canada. E-mail: gordon-ng@merck.com