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The Intracellular Loops of the GB2 Subunit Are Crucial for G-Protein Coupling of the Heteromeric γ-Aminobutyrate B Receptor

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ABSTRACT

The γ-aminobutyrate B ($GABA_B$) receptor is the first discovered G-protein-coupled receptor (GPCR) that needs two subunits, GB1 and GB2, to form a functional receptor. The GB1 extracellular domain (ECD) binds GABA, and GB2 contains enough molecular determinants for G-protein activation. The precise role of the two subunits in G-protein coupling is investigated. GB1 and GB2 are structurally related to the metabotropic glutamate, Ca^{2+}-sensing and other family 3 GPCRs in which the second (i2) as well as the third (i3) intracellular loop play important roles in G-protein coupling. Here, the role of the i2 loops of GB1 and GB2 in the $GABA_B$ receptor ability to activate Gα-proteins is investigated. To that aim, the i2 loops were swapped between GB1 and GB2 heptahelical domains (HDs), either in the wild-type subunits or in the chimeric subunits GB1/2 that contain the ECD of GB1 and the HD of GB2. The effect of an additional mutation within the i3 loop of GB2 that prevents coupling of the heteromeric receptor was also examined. Combinations of interest were found to be correctly addressed at the cell surface and to assemble into heteromers. Taken together our data revealed the following new information on the G-protein coupling of the heteromeric $GABA_B$ receptor: 1) the i2 loop of GB2 within the GB2 HD is required for the heteromeric $GABA_B$ receptor to couple to G-proteins, whereas the i2 loop of GB1 is not; 2) the presence of the i2 loop of GB2 within the GB1 HD is not sufficient to allow coupling of GB1; 3) the GB2 HD activates the Gqi9 protein whether it is associated with the GB2 or GB1 ECD; 4) in the combination with two GB2 HDs, each is able to couple to G-proteins; and finally, 5) the use of mutations in i2, i3, or both within the GB2 HD brings evidence for the absence of domain swapping enabling the exchange of region including i2 and i3 between the subunits.

The main inhibitory neurotransmitter γ-aminobutyrate (GABA) activates two types of receptors, the ligand-gated Cl^--channel receptors ($GABA_A$ and $GABA_C$ receptors) and the metabotropic $GABA_B$ receptor. The later belongs to a large family of receptors coupled to G-proteins. $GABA_B$ receptors modulate synaptic transmission by regulating the activity of Ca^{2+} or K^+ channels (Sodickson and Bean, 1996). $GABA_B$ receptors also inhibit the activity of adenylyl cyclase (for a review see Kerr and Ong, 1995). Most of these actions of the $GABA_B$ receptors are mediated by pertussis toxin-sensitive G-proteins, Go or Gi.

Our understanding of GPCR coupling to G-proteins has always been based on the assumption of a monomeric form of the receptor. However, recent data suggest that at least some GPCRs may function in a dimeric (or multimeric) form (Angers et al., 2002). Moreover, mGlu and Ca^{2+}-sensing receptors have been shown to function as homodimers (Bai et al., 1998). The functional importance of this dimerization is still not fully elucidated. Dimerization of unrelated receptors can lead to a novel receptor with unique pharmacological properties, profile of G-protein coupling, and association with other signaling pathways (Bouvier, 2001). These observations raise a number of questions about the respective roles of the two subunits in a dimeric GPCR.

The structure of the $GABA_B$ receptor was elusive until

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ABBREVIATIONS: GABA, γ-aminobutyrate; GPCR, G-protein-coupled receptors; mGlu receptors, metabotropic glutamate receptors; ECD extracellular domain; HD, heptahelical domain; i2 loop, second intracellular loop; i3 loop, third intracellular loop; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; IP, inositol phosphates; XL665, allophycocyanin.

recently when the cloning of two $GABA_B$ receptor cDNAs, GB1 (Kaupmann et al., 1997) and GB2, was reported (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). Coassembly of both GB1 and GB2 proteins was found to be essential for an efficient coupling of the receptor to G-proteins. Both proteins are related to the "family 3" of GPCRs represented by the mGlu, Ca^{2+}-sensing, some taste, and putative vomeronasal receptors (Bockaert and Pin, 1999). A unique feature of family 3 receptors is their large N-terminal extracellular domain (ECD), which is structurally related to bacterial periplasmic binding proteins and constitute their ligand recognition domain (Galvez et al., 1999; Malitschek et al., 1999). Ligand binding into this Venus flytrap like module causes activation of the heptahelical domain (HD). How the activation of extracellular domain further transmitted to the HD is not known yet. Anyhow, mGlu receptors are homodimers, and it has been proposed that the dimeric structure of the receptor is crucial for activation of the receptor (Kunishima et al., 2000).

Recently, several studies revealed some specific roles of each subunit of the $GABA_B$ receptor. GB1 subunit contains the extracellular N-terminal region responsible for ligand binding, but the corresponding region of GB2 subunit, although it is unlikely to bind GABA, is required for high affinity binding of agonists on GB1 (Galvez et al., 2000). Moreover, GB2 by interacting with GB1 at the level of its C-terminal tail, masks an endoplasmic reticulum retention signal such that only heterodimeric receptors are correctly inserted in the plasma membrane (Margeta-Mitrovic et al., 2000; Calver et al., 2001; Pagano et al., 2001). Finally, it has been shown recently that the GB2 HD contains enough molecular determinants for coupling to G-proteins, because a G-protein activation can be detected with a $GABA_B$ receptor combination containing GB2 HDs only (Galvez et al., 2001). However, the exact role of each subunit in G-protein recognition and activation by the heterodimeric $GABA_B$ receptor is still unknown.

Within the family 3 GPCRs, the second intracellular (i2) loop plays a critical role for the selective interaction with G-proteins, whereas the i3 loop is important for coupling efficacy (Pin et al., 1994; Gomeza et al., 1996; Francesconi and Duvoisin, 1998; Chang et al., 2000). The homodimeric structure of mGlu receptors results in receptor complexes in which each unit contains pairs of identical intracellular loops. However, in the case of the $GABA_B$ receptor, the two i2 loops (that of GB1 and that of GB2) differ substantially from each other. This situation offers a nice opportunity to identify the role of two i2 loops in a dimeric receptor. Are both i2 loops required for the recognition and activation of the G-protein? Do they direct the coupling to different G-proteins?

To that end, several chimeric proteins were constructed in which the i2 loops between GB1 and GB2 were swapped. To investigate the roles of other intracellular portions, the GB2 L686P mutant recently described was used (Duthey et al., 2002). These chimeric proteins were functionally expressed in a heterologous system with wild-type subunits. Construct GB1/2, in which the ECD of GB1 subunit is connected to the HD of GB2, and mutants of this chimera were employed to dissect further the functional role of each HD.

The present data demonstrate first that within the wild-type heterodimer receptor complex, the two subunits do not play the same role in activation of G-proteins. Our data show that the i2 loop of GB2 is required for coupling of the heterodimeric receptor to G-proteins, whereas that of GB1 is not. Second, within the recombinant receptor that includes HD from GB2 only, it seems that each HD activates G-proteins. The HD of GB2 that is capable of coupling to G-proteins has to include both the second and the third intracellular loops intact. By introduction of the i2 loop from GB1 or by mutating the i3 loop, the subunit capability of coupling is lost. Interestingly, mutated region cannot be supplied from other functional subunit by mechanism of domain swapping. These observations shed more light on the role of GB2 and on the role of each HD in a dimeric receptor. Thus, it will be useful for other studies aimed at explaining the nature of dimerization of GPCRs.

Experimental Procedures

Materials. Chemicals including GABA were obtained from Sigma-Aldrich Chimie SARL (L'Isle d'Abeau Chesnes, France) unless otherwise indicated. Serum, culture media, and other solutions used for cell culture were from Invitrogen SARL (Cergy Pontoise, France). The plasmids expressing $GABA_B$ receptor subunits and their chimeras were described previously (Galvez et al., 2001; Duthey et al., 2002). The $G\alpha qi9$ and $G\alpha qo$ proteins (Liu et al., 1995) were kindly provided by Dr. Bruce Conklin (The Gladstone Institute, San Francisco, CA). The mutagenesis was done by introducing silent restriction sites by quick exchange technology (Stratagene, Amsterdam, The Netherlands) at both predicted ends of the second intracellular loops of GB1 and GB2. Swapping of the loop was done using the sites *Nru*I in the N-terminal portion and *Hin*dIII at the C-terminal portion of the i2 loops (see Fig. 1).

Culture and Transfection of Human Embryonic Kidney (HEK 293) cells. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen SARL) supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin, 100 U/ml final). Electroporation was performed in a total volume of 300 μl with 6 μg of carrier DNA, $GABA_B$-subunit plasmid DNA (2 μg), Gα-subunit plasmid DNA (2 μg), and 10 million cells in electroporation buffer (50 mM K_2HPO_4, 20 mM CH_3COOK, 20 mM KOH, pH 7.4). After electroporation (260 V, 1 mF, Bio-Rad Gene Pulser electroporator; Bio-Rad Laboratories, Hercules, CA), cells were resuspended in DMEM supplemented with 10% fetal calf serum and antibiotics, and split in 12-well clusters (Falcon, Paris, France) (10 million cells per 12 wells) previously coated with poly-L-ornithine (15 μg/ml; M_r 40,000; Sigma-Aldrich Chimie SARL) to favor adhesion of the cells.

Determination of Inositol Phosphates Accumulation. The procedure used for the determination of IP accumulation in transfected cells was adapted from previously published methods (Berridge and Irvine, 1984). Cells were washed 2 to 3 h after electroporation and incubated for 14 h in DMEM (Invitrogen SARL) containing 0.4 μCi/ml [*myo*-^{3}H]inositol (23.4 Ci/mol; PerkinElmer Life Sciences, Paris, France). Cells were then washed two times with HEPES-buffered saline (146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 0.1% glucose, 20 mM HEPES, pH 7.4), and LiCl was added to a final concentration of 10 mM. The agonist was applied 5 min later and left for 30 min at 37°C. Replacing the incubation medium with 0.5 ml of perchloric acid (5%) stopped the reaction, and the clusters were kept on ice for 30 min. Supernatants were recovered, and the IPs were purified on Dowex columns (Bio-Rad) (Berridge and Irvine, 1984) Total radioactivity remaining in the membrane fraction was counted after treatment with 10% Triton X-100, 0.1 N NaOH for 30 min (room temperature) and used as standard. Results are expressed as the amount of IP produced over the radioactivity present in the membranes. The dose-response curves were fitted according to the equation $y = [(y_{max} - y_{min})/1 + (x/EC_{50})^n] + y_{min}$ using the KaleidaGraph program (Abelbeck Software, Reading, PA).

Ligand Binding of Receptors on the Cell Surface. Binding assay was done on intact cells to measure properties of receptors that pass the plasmalemma of HEK 293 cells. Cells were plated in 24-well plates after transfection (done as in previous experimental procedure) in density about 10 million cells per plate. On the next day, they were put on ice and washed three times with binding buffer (20 mM Tris-HCl, pH 7.4, 118 mM NaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl, and 1.8 mM $CaCl_2$). They were incubated in the presence of 0.1 nM ^{125}I-CGP64213 with or without competitive-unlabeled ligand for 3 h at 4°C. The compound ^{125}I-CGP64213 does not pass the cell membrane. The incubation was terminated by washing three times with ice-cold binding buffer. Cells were then disrupted by 0.1 M NaOH and bound radioactivity was counted. Nonspecific binding was determined in the presence of GABA (1 mM).

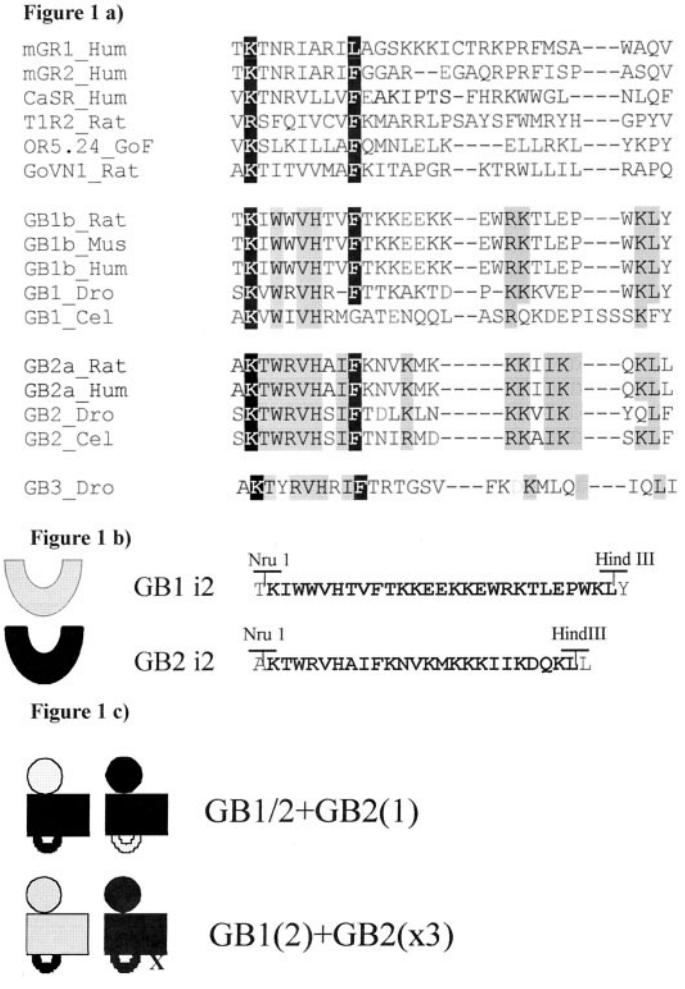

Fig. 1. a, alignment of the sequences corresponding to the i2 loops of receptors from family 3 GPCR including the GB1 and GB2 sequences from different species. Residues highlighted in black are those conserved in all family members. The residues in gray are those conserved in all GB1 or GB2 i2 loops from different species. GB3 Dro correspond to a cDNA that has been recently isolated from *Drosophila melanogaster* and which share similarities with GB1 and GB2 but no function discovered yet (GenBank accession no. AF318274). b, position of silent restriction sites that were used to swap the i2 loop between GB1 and GB2 (*Nru*I and *Hin*dIII). c, example of nomenclature of the wild type and chimeric subunits used in this study. In the upper combination, GB1/2+GB2/(1) is composed of ECD from GB1 followed by HD-GB2 and coexpressed with GB2 with the i2 loop of GB1. Below, in the combination GB1(2)+GB2(xi3), the GB1 subunit carries the i2 loop from GB2, and the GB2 protein is mutated L686P within the i3 loop (see also Table 1). Hum, Human; GoF, Goldfich; Dro, *D. melanogaster*; Cel, *C. elegans*.

Measurement of Inhibition of cAMP Formation. Production of cAMP was measured as follow. The cells were treated and electroporated as described above. The agonist was applied after two washes with Krebs buffer for 30 min at 37°C. The stimulation of receptors was then stopped by lysis of the cells by 0.5% Triton X-100 in distilled water for 30 min at room temperature. The supernatant was recovered and competitive immunoassay using labeled anti-cAMP antibodies (with cryptate), and cAMP labeled with XL665 as competitor was carried for 1 h at 4°C using the cAMP kit (CIS Bio International, Paris, France). The transfer of energy between the cryptate and the XL665 was counted using the RUBYstar reader (BMG Labtechnologies, Durham, NC).

Results

Construction and Functional Expression of Chimeric GB1 and GB2 Proteins.

The alignment of sequences showed there are substantial differences between the second intracellular loops (i2 loops) of GB1 and GB2 and the other members of the family 3 GPCR (Fig. 1a). This suggests that GB1 and GB2 do not couple similarly to G-proteins. To examine the role of i2 loops from GB1 and GB2, these were swapped between the two subunits. Chimeric GB1 subunit that contains the i2 loop of GB2, called GB1(2), and the reciprocal GB2 chimera with the second intracellular loop of GB1, called GB2(1), were generated (Table 1 and Fig. 1).

The chimeras were tested either alone or in several combinations with wild-type subunits or with other chimeric subunits that were described in our previous studies (Fig. 1) (Galvez et al., 2001). The GB2(x3) mutants, in which the L686P mutation was introduced within the third intracellular loop, was described previously (Duthey et al., 2002). As a control that all combinations were expressed, assembled, and targeted at the cell surface when transfected into HEK 293 cells, we performed binding experiment on intact cells with the impermeant GB1-specific radioligand ^{125}I-CGP64213 (Table 2).

The Second Intracellular Loop of GB1 Is Not Required for G-Protein Coupling

GB1(2)+GB2. When we coexpressed the wild-type receptor with a chimeric Gαqi9 protein in which the extreme C-terminal 9 amino acids of Gi replace those in Gαq (Liu et al., 1995), a GABA$_B$ receptor coupling to phospholipase C can be measured as inositol phosphate accumulation (Franek et al., 1999). The GB1 subunit with the i2 loop of GB2]chimera GB1(2); Fig. 1c[expressed alone did not respond to GABA (data not shown). However, the coexpression of this chimera GB1(2) with the

TABLE 1
Description of the different constructs used in this study
See also Fig. 1.

Name	ECD	HD	i2	i3
GB1	1	1		
GB1(2)	1	1	2	
GB1(x3)	1	1		I798P
GB1/2	1	2		
GB1/2(1)	1	2	1	
GB1/2(x3)	1	2		L686P
GB2	2	2		
GB2(1)	2	2	1	
GB2(x3)	2	2		L686P
GB2(1, x3)	2	2	1	L686P

wild-type GB2 subunit formed a functional receptor complex (Fig. 2a). Potency and maximal effects of GABA on this combination are similar to those obtained with the wild-type receptor, showing that combination GB1(2)+GB2 behaves like the wild-type GB1+GB2 (Fig. 2b).

This demonstrates that the GB1 i2 loop is not crucial in respect to G-protein coupling in our system. Moreover, the presence of two GB2 i2 loops in one receptor complex did not alter the coupling.

GB2 i2 Loop Is Required for G-Protein Coupling

GB1+GB2(1). The question about the function of the i2 loop of GB2 in the receptor complex was addressed by replacing the i2 loop of the GB2 subunit with that of GB1. The coexpression of chimera GB2(1) with the wild-type GB1 subunit did not result in the formation of a functional receptor (Fig. 2a). This is not due to a miss-folding of the protein GB2(1) as it was capable of association with and trafficking of the GB1 subunit to the cell surface as detected with the membrane impermeable radioligand ^{125}I-CGP64213 (Table 2). Moreover, displacement of this radioligand with GABA revealed a high affinity state similar to that measured on the wild-type combination (Table 2). This control proves that the ECDs of the two subunits interact like those in the wild-type combination. These data further illustrate the importance of the GB2 HD, and its i2 loop is G-protein coupling of the heteromeric $GABA_B$ receptor.

The i2 Loop from GB2 in the Environment of GB1 Is Not Sufficient for G-Protein Coupling of GB1

GB1(2)+GB2(1). We then examined the possibility that GB1 subunit is not capable of G-protein coupling because of amino acid sequence of its i2 loop. The coexpression of the chimeras did not lead to a functional receptor as no significant formation of inositol phosphate was detected upon stimulation with GABA (Fig. 2a). However, the chimeric proteins were expressed and correctly inserted into the plasma membrane, and the allosteric interaction between the GB1 and GB2 ECDs is like that in the wild-type combination as can be seen from binding experiments on intact cells with ^{125}I-CGP64213 and the determination of GABA affinity (Table 2). Thus, the i2 loop of GB2 is not sufficient to allow the coupling of GB1 HD to Gqi9.

The i2 Loop of GB2 Is Crucial for Coupling to Native Gαi-Proteins.

Because the natural partners of $GABA_B$ receptor are pertussis toxin sensitive Gi/*o*-proteins, we next examined the coupling of various $GABA_B$ receptor combinations to the inhibition of adenylyl cyclase. In cells expressing the wild-type $GABA_B$ receptor combination, GABA (Table 3) as well as baclofen (not shown) inhibit forskolin stimulated cAMP formation. A similar inhibition was also obtained with the GB1(2)+GB2 but not with the GB1(2)+GB2(1) (Table 3). This clearly illustrates that the i2 loop of GB2 is important not only for the coupling of the heteromeric receptor to Gqi9 but also to the native G-protein.

Each HD in the GB1/2+GB2 Recombinant Receptor Is Capable of Coupling to G-Proteins

GB1/2+GB2(1), GB1/2(1)+GB2, GB1/2(1)+GB2(1). Although the combination in which both subunits contain a GB2 HD (GB1/2+GB2) is functional, the role of each GB2 HD within this recombinant receptor in G-protein coupling is not fully understood. This recombinant complex has lower coupling efficacy than the wild-type receptor, but the G-protein

TABLE 2

The maximal specific binding is expressed as a percentage of the maximal specific binding of the W_T combination in the same experiments

The data are the mean ± S.E.M. of the values determined in at least three independent experiments. The affinity values (K_i, determined as described under *Materials and Methods*) of GABA were determined from displacement of ^{125}I-CGP64213 binding on intact cells expressing the indicated combinations. The K_i values are means ± S.E.M. of at least three independent experiments and are determined for each combination taking into account the K_d value for CGP64213 calculated from cold Scatchard plots. The ratios are the ratios of the GABA K_i divided by the GABA K_i obtained with the combination WT, and the ratios presented are the mean of the ratio determined in each experiment. The ASA mutant of GB1 that carries mutation within the C terminus that allows it to pass the endoplasmic reticulum (Pagano, et al., 2001) is used to compare the binding of GB1 ECD without the allosteric modulation by GB2.

Combinations	125I-CGP64213 BiN.D.ing			
	Maximal ^{125}I-CGP64213 BiN.D.ing	GABA K_i	Hill Coefficient	Ratio K_i/K_i(WT)
	%/WT	*μM*		
GB1+GB2	100.0 ± 0.0	3.33 ± 0.66	1.1 ± 0.1	1.0 ± 0.0
GB1ASA	108.7 ± 8.8	16.88 ± 3.22	1.0 ± 0.2	6.1 ± 2.5
GB1/2	83.8 ± 7.0	14.15 ± 2.18	1.3 ± 0.1	4.9 ± 1.7
GB1/2(1)	101.2 ± 23.6	11.68 ± 1.21	1.0 ± 0.2	3.9 ± 1.1
GB1(2)+GB2	120.0 ± 38.8	N.D.	N.D.	N.D.
GB1+GB2(1)	134.9 ± 35.6	4.57 ± 0.95	1.3 ± 0.3	1.5 ± 0.4
GB1(2)+GB2(1)	105.4 ± 1.5	N.D.	N.D.	N.D.
GB1/2+GB2	94.3 ± 4.8	N.D.	N.D.	N.D.
GB1/2+GB2(1)	92.7 ± 23.6	3.87 ± 0.90	1.1 ± 0.1	1.1 ± 0.1
GB1/2(1)+GB2	74.5 ± 23.2	4.97 ± 2.39	1.5 ± 0.2	1.3 ± 0.7
GB1/2(1)+GB2(1)	67.1 ± 16.1	2.65 ± 1.46	1.5 ± 0.5	0.7 ± 0.3
GB1+GB2(x3)	129 ± 3.5	N.D.	N.D.	N.D.
GB1/2+GB2(x3)	96.9 ± 3.5	N.D.	N.D.	N.D.
GB1/2(1)+GB2(x3)	92.3 ± 2.5	N.D.	N.D.	N.D.
GB1/2x+GB2	106.7 ± 2.2	N.D.	N.D.	N.D.
GB1/2x+GB2(x3)	89.4 ± 3.9	N.D.	N.D.	N.D.
GB1/2x+GB2(1)	117.3 ± 3.3	N.D.	N.D.	N.D.
GB1/2(1)+GB2(1,x3)	83.5 ± 0.5	N.D.	N.D.	N.D.
GB1/2(x3)+GB2(1,x3)	112.2 ± 1.4	N.D.	N.D.	N.D.
GB1/2+GB2(1,x3)	157.6 ± 10.4	N.D.	N.D.	N.D.
GB1+GB2(1,x3)	129.2 ± 5.8	N.D.	N.D.	N.D.

N.D., not determined; WT, wild type; ASA, Ala-Ser-Ala.

coupling selectivity is maintained (Galvez et al., 2001). Are both HDs required for coupling? Is only one HD able to couple within the dimer? Or are both HD able to couple G-proteins? These questions are relevant not only for our understanding of the GABA$_B$ receptor functioning but also for our understanding of the other family 3 GPCRs, which are homodimers in many cases.

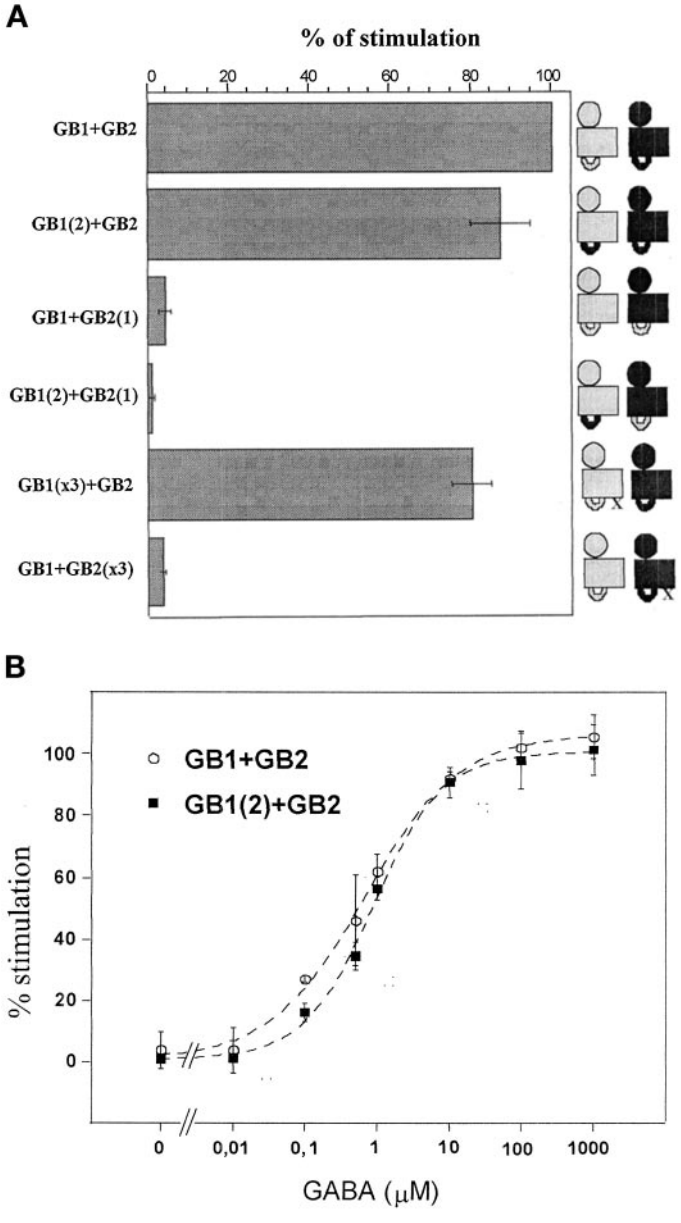

Fig. 2. Functional analysis of GABA$_B$ receptor combinations in which the i2 loop from GB2 replaces that of GB1 [chimera GB1(2)] or the reverse chimera GB1(2) and the mutants carrying mutation in the i3 loops of GB2 [mutants GB1(xi3) and GB2(xi3), respectively]. The receptor proteins were expressed in HEK 293 cells and coupling to Gqi proteins was measured as IP accumulation (see *Experimental Procedures*). a, the maximal responses after stimulation with 1 mM GABA. Wild-type combination GB1+GB2 is 100%. The basal activities (values represented as IP3/total radioactivity) were 8.1 ± 1.5 (GB1+GB2), 4.1 ± 0.4 [GB1(2)+GB2], 2.7 ± 0.1 [GB1+GB2(1)], 2.9 ± 0.5 [GB1(2)+GB2(1)], 9.2 ± 1.3 [GB1(x3)+GB2], 2.5 ± 0.2 [GB1+GB2(x3)], and the stimulation of GB1+GB2 (wild-type receptor) by 1 mM GABA was (41.1 ± 1.2). b, dose-response curves measured with increasing concentrations of GABA on cells expressing the indicated combination of subunits. Data are means ± S.E.M. of three independent experiments performed in triplicates.

To answer this question, we examined the coupling to Gqi9 of the GB1/2+GB2 combination in which one of the two GB2 HDs bears the i2 loop of GB1 [GB1/2+GB2(1) or GB1/2(1)+GB2] (Fig. 3a). These complexes were able to couple to G-proteins but with decreased maximal effect compared with the GB1/2+GB2, even though they were expressed at a similar level (Table 2). A total absence of coupling was observed if both subunits bear the GB1 i2 [GB1/2(1)+GB2(1)] (Fig. 3a and Table 2). These data are consistent with one intact GB2 HD within the dimeric receptor being necessary for coupling to G-proteins.

The L686P Mutation in the i3 Loop of GB2 Confirms That Each HD in the GB1/2+GB2 Recombinant Receptor Is Capable of Coupling to G-Proteins

GB1/2+GB2(x3), GB1/2(x3)+GB2 and GB1/2(x3)+GB2(x3), GB1/2(1;x3)+GB2 and GB1/2(1;x3)+GB2. The experiments described above suggest that both GB2 HDs can couple to G-proteins in the GB1/2+GB2 combination. To further confirm this, we analyzed the functioning of GB1/2+GB2 combination in which the coupling to G-proteins of one (or both) GB2 HD is prevented by the L686P mutation in the i3 loop [GB2(x3) mutant] (Duthey et al., 2002), with or without the i2 loop of GB1.

The combinations GB1/2+GB2(x3) and GB1/2+GB2(1;x3) did couple less efficiently to G-proteins than the GB1/2+GB2 but as efficiently as the combination GB1/2+GB2(1) (Fig. 3). Similar data were obtained with the i3 mutation introduced in the GB1/2 or GB1/2(1) chimera coexpressed with GB2. As expected, when both HD domains carried the mutated third intracellular loop [GB1/2(x3)+GB2(x3)], no coupling to G-protein was detected (data not shown). Again, the lower response, or the absence of response, is not due to a lower level of expression of the indicated combinations nor to the absence of allosteric interaction between the subunits (Table 2). Taken together, these data further demonstrate that both GB2 HDs couple to G-proteins in the GB1/2+GB2 dimer.

Domain Swapping Is Unlikely to Occur between the Two HDs in GB1/2+GB2

GB1/2(1)+GB2(x3) and GB1/2(x3)+GB2(1). We next asked whether within the GABA$_B$ receptor heterodimer transmembrane, domains can be swapped between the HDs. To test this possibility, we expressed combinations of the receptors in which at least one intact i2 loop and one intact i3 loop can be found, but there is at least one of such mutation per subunit. If one subunit could subsidize portion of the partner molecule within the complex so it will gain ability to couple to G-protein, this would suggest that domain swapping within the receptor complexes can occur. To investigate

TABLE 3

Expression of GB1 and GB2 in HEK 293 cells leads to functional receptor that is upon GABA application capable of activating Gi proteins.

The inhibition of adenylyl cyclase can be measured as decrease in cAMP formation. The mutated combination GB1(2) was compatible with the Gi protein coupling in this assay. On the other hand, the GB2(1) mutant coexpressed with GB1 or GB1(2) did not activate Gi-proteins. The values are means ± S.E.M. of at least three independent determinations.

Combinations	% of cAMP Inhibition
GB1+GB2	13.4 ± 2.7
GB1(2)+GB2	22.6 ± 6.5
GB1(2)+GB2(1)	0 ± 0

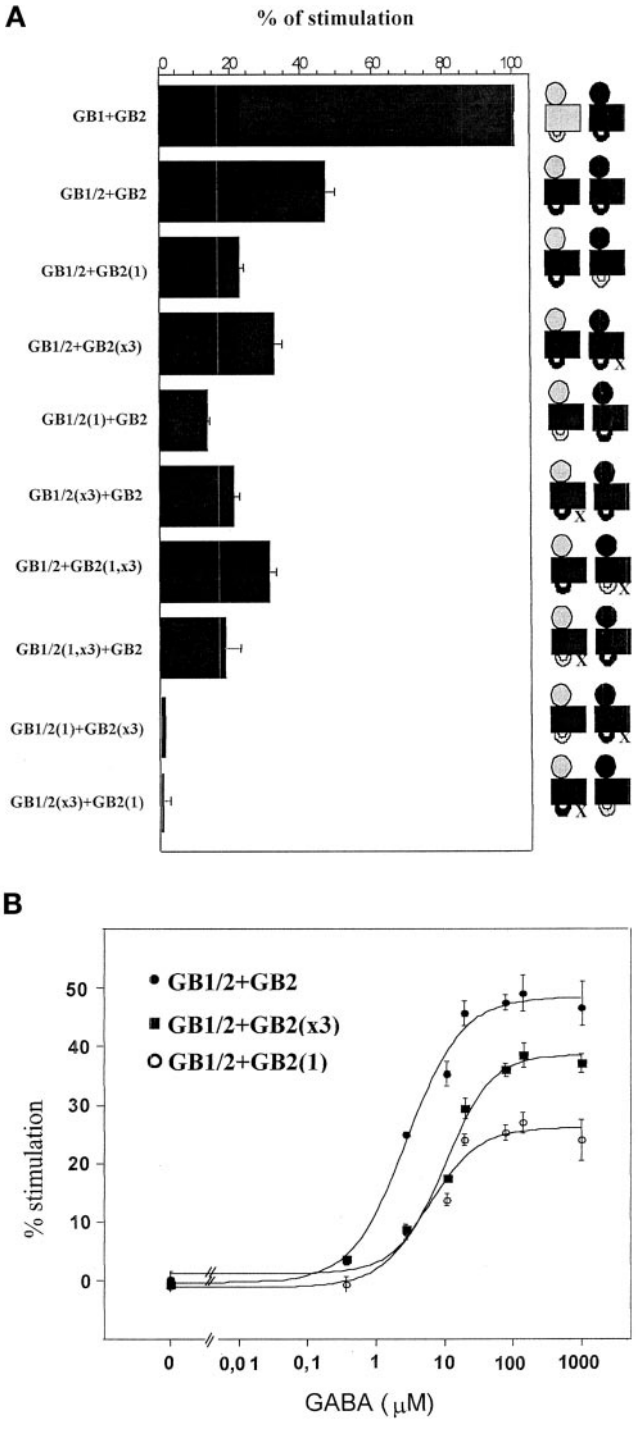

Fig. 3. a, functional analysis of $GABA_B$ receptor combinations GB1/2+GB2 [originally described at (Galvez et al., 2000)] and their i2 loop (i2 loop from GB1 replaces that of GB2) and i3 loop (L686P) mutants. IP production was measured after stimulation with a saturating GABA (1 mM). Data are expressed as the percentage of the response obtained with the wild-type combination and are means ± S.E.M. of three independent experiments performed in triplicates. The basal activities (values represented as IP3/total radioactivity) were 3.8 ± 0.3 for (GB1/2+GB2), 3.2 ± 0.2 [GB1/2+GB2(1)], 3.5 ± 0.2 [GB1/2+GB2(x3)], 3.5 ± 0.4 [GB1/2(1)+GB2]; 4.1 ± 0.5 [GB1/2(x3)+GB2], 3.1 ± 0.3 [GB1/2+GB2(1,x3)], 2,1 ± 0,5 [GB1/2(1,x3)+GB2], 3.0 ± 0.2 [GB1/2(1)+GB2(x3)], 3.0 ± 0.2 [GB1/2(x3)+GB2(1)]. The basal activity of GB1+GB2 was 8.1 ± 1.5 and stimulation by 1 mM GABA 41.1 ± 1.2 (=100%). b, dose-response curves measured with increasing concentrations of GABA on cells expressing the indicated combination of subunits. Data are means ± S.E.M. of three independent experiments performed in triplicates.

this possibility, we coexpressed subunits in which alternatively the second or the third intracellular loops were mutated within the HD of GB2 so that one mutation is within one molecule strictly, and at the same time, each subunit carries one or the other mutation not compatible with G-protein coupling. As our results clearly show, coexpression of GB1/2(1)+GB2(x3) or GB1/2(x3)+GB2(1) led to receptor complexes that did not couple to G-proteins, even though they are correctly expressed (Fig. 3a and Table 2). We therefore conclude that these receptors are dimers in which the protomers cannot exchange portions of their HDs, at least not the portions around or between the i2 and i3 loops.

Discussion

Recently, many GPCRs have been found to form dimers (Bouvier, 2001), either homo- or heterodimers. In some cases, heterodimers have been shown to display specific properties not shared by any receptor expressed alone. This could be a different pharmacological profile, different G-protein-coupling selectivity, or different desensitization properties of such complexes. However, the specific role of each subunit within a dimeric GPCR in G-protein coupling is not known. In the present study, we took advantage of the heteromeric nature of the $GABA_B$ receptor constituted of the GB1 and GB2 subunits to start elucidating this question.

In a previous study, we reported that the ECDs of both GB1 and GB2 were required for activation of the $GABA_B$ receptor by agonists (Galvez et al., 2001). Accordingly, only combinations of $GABA_B$ subunits containing both GB1 and GB2 ECDs were tested in the present study. Our data illustrate the different role of each heptahelical domain and the intracellular loops within a dimeric receptor and their importance for proper coupling to G-protein. These data suggest that the GB1 subunit does not couple to G-proteins or at least not by the same mechanism as the GB2 subunit does. The intracellular regions of the HDs of mGlu and CaS receptors, and especially their i2 loop, play an important role in G-protein-coupling selectivity (Pin et al., 1994; Gomeza et al., 1996; Francesconi and Duvoisin, 1998; Chang et al., 2000). The i2 loop of GB1 cannot replace that of GB2 in the process of G-protein activation. Alignment of the i2 loops of family 3 GPCRs revealed only two highly conserved residues, a Lys residue at the bottom of TM3 and a Phe (or Leu) eight residues later (Fig. 1). Site directed mutagenesis of the i2 loop of the CaS receptor revealed that the Phe was crucial for G-protein coupling (Chang et al., 2000). As shown in Fig. 1, these two residues are conserved in GB2 and GB1 (except that of the *Caenorhabditis elegans* GB1) i2 loops (Fig. 1) and cannot therefore explain the differential coupling of these two subunits observed here. It has also been shown that the two-third C-terminal region of the i2 loop of mGlu1 was playing a critical role in specifying Gq coupling (Pin et al., 1994). As shown in Fig. 1, the two-third N-terminal sequence of the i2 loops of GB1 and GB2 are quite different, possibly explaining the difference in the G-protein-coupling ability noticed here. Interestingly, whereas the i2 loop of GB2 is well conserved from *C. elegans* to mammals, this is not the case for the i2 loop of GB1 in which many acidic residues can be found in the mammalian sequences. Indeed, a recent study that appeared after submission of this article revealed that such acidic residues prevent $GABA_B$ receptor coupling to

G-proteins when introduced in the i2 loop of GB2 (Robbins et al., 2001). Although the i2 loop of GB2 is shown to be critical for coupling to G-proteins, its presence within the GB1 HD is not sufficient to allow coupling of the GB1 HD. It has been shown in the structurally related mGlu receptors that although the i2 loop of the phospholipase C-coupled mGlu1 was necessary for coupling to Gq, it was not sufficient, in that it did not couple to Gq alone in the environment of HD from mGluR3. Indeed, the presence of an additional intracellular portion of mGlu1 was also required (i.e., either i1, i3, or C-terminal tail). Similarly, the other intracellular loops of GB2, i1 (Margeta-Mitrovic et al., 2001a) and i3 (Margeta-Mitrovic et al., 2001a; Duthey et al., 2002) are important for proper coupling to G-protein. Although the C-terminal tails of the GB subunits may be involved, their deletion does not prevent coupling (Margeta-Mitrovic et al., 2000; Calver et al., 2001; Pagano et al., 2001), highlighting the importance of the intracellular loop of GB2 for G-protein coupling.

Taken together, our data further support the proposal that, like in rhodopsin-like receptors, the i2 and i3 loops of family 3 GPCRs form a binding pocket for the C-terminal end of the G-protein α subunit. In addition, our data demonstrate that the GB2 HDs within the GB1/2+GB2 combination cannot exchange their transmembrane domains in such a way that at least one G-protein α-subunit binding pocket is restored. This observation also explains why GB1(2)+GB2(1) is not capable of G-protein activation, despite the fact that such combination includes all $GABA_B$ receptor portions. This suggests that domain swapping as proposed by some authors (Dean et al., 2001) does not occur between the GB subunits.

Our data, as well as those reported recently (Margeta-Mitrovic et al., 2001a; Robbins et al., 2001; Duthey et al., 2002) are consistent with GB1 HD not being able to activate a G-protein. However, none of these studies examined the possible influence of the ECD on the G-protein-coupling ability of the HD. Indeed, it remained possible that the GB1 ECD prevents coupling of its associated HD and that in contrast the GB2 ECD allows the coupling of its associated HD. Our present data rule out this possibility. The GB2 HD can activate G-protein whether it is associated with the GB1 ECD [such as in combinations GB1/2+GB2(1) and GB1/2+GB2(xi3) and GB1/2+GB2(1,xi3)] or with the GB2 ECD. Such an observation is consistent with the recent activation mechanism proposed for family 3 GPCRs based on the crystal structure of the dimer of mGlu1 ECD with and without glutamate (Kunishima et al., 2000) (see Fig. 4) and based on other data obtained with the $GABA_B$ receptor (Galvez et al., 2001; Margeta-Mitrovic et al., 2001b). According to this model, binding of one agonist in one ECD is sufficient to induce a large conformational change of the dimer of ECDs such that their respective C-terminal ends become closer. This probably forces the two HDs to interact differently, leading to the stabilization of the active state of the GB2 HD. Obviously this seems independent on the ECD connected to the GB2 HD.

If the GB2 HD contains the G-protein α-subunit binding site, what is the respective role of these two sites found in the GB1/2+GB2 combination? Our data show a significant decrease in the maximal response when either one of these two sites is mutated, either by the replacement of the i2 loop by that of GB1 or by the L686P mutation in the i3 loop. In addition, a total loss of coupling was observed when both sites are mutated. Accordingly, both sites in a receptor with a homodimeric HD can couple to the G-protein, resulting in an increased coupling efficacy. Accordingly, for homodimeric GPCRs, like the other family 3 receptors, the ability of both subunits to couple to the same G-protein is probably important for their efficient coupling. In agreement with this possibility, a combination of two calcium-sensing receptors in which one subunit is mutated such that it does not activate the G-protein couples less efficiently than the wild-type homodimer (Bai et al., 1999).

What is the role of GB1 in G-protein coupling of the heteromeric receptor? Our data indicate that the replacement of the GB1 i2 loop by that of GB2 does not alter coupling. Similarly, after submission of the present manuscript, three reports revealed that mutations in either the i1, i2, or i3 loop of GB1 do not alter coupling of the heteromeric $GABA_B$ receptor (Margeta-Mitrovic et al., 2001a; Robbins et al., 2001; Duthey et al., 2002). Accordingly, one can conclude that the GB1 subunit does not activate the G-protein by interacting with the α subunit. However, we previously reported that the GB1/2+GB2 combination, although it activates a same set of G-proteins as the GB1+GB2 heteromeric receptor (Galvez et al., 2001), couples less efficiently than the wild-type. It is therefore possible that the GB1 HD contacts the G-protein at a site different from the C-terminal end of the α subunit, possibly at the level of either the β or the γ subunit. Alternatively, the GB1 HD may stabilize a different conformation of the GB2 HD that couples more efficiently to the G-protein, consistent with the model proposed in Fig. 4. More work will be necessary to discriminate between these two possibilities. In addition, it remains possible that the GB1 HD plays an additional role in the $GABA_B$ receptor function than just to enhance G-protein-coupling efficacy. Indeed, the demonstration that the GB1 C-terminal tail interacts with the transcription factors ATF4 and ATFx (White et al., 2000) suggests that this subunit may be involved in G-protein-independent transduction cascades of the $GABA_B$ receptor.

In conclusion, our data show that within the heteromeric $GABA_B$ receptor, the GB2 subunit is the one that couples to G-proteins. How can the binding of GABA within the GB1 ECD lead to the stabilization of the active state of the GB2 HD? The recent determination of the crystal structure of the dimeric mGlu1 ECDs sheds some light on this issue. Indeed,

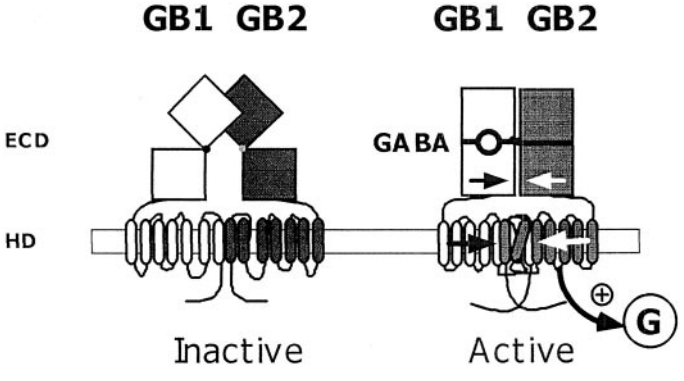

Fig. 4. Schematic representation of the putative activation mechanism of the heteromeric $GABA_B$ receptor. According to this model, agonist binding in GB1 ECD induces a change in conformation of the dimer of ECDs and thus forces the two HDs to interact with each other differently, leading to the stabilization of the active GB2 HD, whether it is linked to the GB2 ECD (like in the wild-type receptor) or the GB1 ECD.

agonist binding induces a large conformation change of the dimeric ECD, such that the C-terminal ends of each protomer become closer by more than 20 A. This is likely to stabilize a new conformation of the dimer of HDs leading to the activation of the G-protein. Accordingly, one may propose GABA binding in the GB1 ECD stabilizes a new conformation of the dimeric ECDs, which in turn will stabilize the active conformation of the GB1-GB2 heteromeric HDs, no matter to which HD the GABA binding domain is attached.

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