Signal Transduction by GABA$_B$ Receptor Heterodimers

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GABA$_B$ receptors are G-protein-coupled receptors that mediate inhibition throughout the central and peripheral nervous systems. A single cloned receptor, GABA$_B$R1, which has at least three alternatively spliced forms, appears to account for the vast majority of binding sites in the brain for high-affinity antagonists. In heterologous expression systems GABA$_B$R1 is poorly expressed on the plasma membrane and largely fails to couple to ion channels. A second gene, GABA$_B$R2, which exhibits moderately low homology to GABA$_B$R1, permits surface expression of GABA$_B$R1 and the appearance of baclofen-sensitive K$^+$ and Ca^{++} currents. We review the data that supports a model of the native GABA$_B$ receptor as a heterodimer composed of GABA$_B$R1 and GABA$_B$R2 proteins. New data from mutagenesis experiments are presented that point to amino acid residues on GABA$_B$R1 critical for ligand activation of the heterodimer. The possible role of GABA$_B$R2 in signal transduction is also discussed. The interdependent nature of the two subunits for receptor function makes the GABA$_B$ receptor a useful model to explore the larger significance of GPCR dimerization for G-protein activation.
[Neuropsychopharmacology 23:S41–S49, 2000]
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Gamma-aminobutyric acid (GABA) is an abundant neurotransmitter that mediates inhibition throughout the nervous system via both ligand-gated channels (GABA$_A$ and GABA$_C$ receptors) and G-protein-coupled receptors (GABA$_B$ receptor). GABA$_B$ receptors have both pre- and postsynaptic actions (Bowery 1993; Price et al. 1984). Postsynaptic inhibition of neuronal firing is mediated primarily by the coupling of GABA$_B$ receptors to the activation of inwardly rectifying K$^+$ channels (GIRKs) (North 1989; Gahwiler and Brown 1985; Andrade et al. 1986). Presynaptic inhibition of neurotrans-mitter release by GABA is thought to occur by suppression of any of a number of identified high-threshold Ca^{++} channels (Harayama et al. 1998; Lambert and Wilson 1996; Mintz and Bean 1993; Dolphin and Scott 1987). The physiological importance of GABA$_B$ receptors has been widely appreciated in humans through the use of baclofen (Bowery et al. 1980), a selective agonist at GABA$_B$ receptors that is used clinically for the treatment of muscle spasticity and trigeminal neuralgia. The widespread distribution of GABA$_B$ receptors in both central and peripheral nervous systems reveals their importance in a variety of physiological processes.

There is now a substantial repertoire of ligands, having agonist or antagonist properties, that display high affinity and selectivity for GABA$_B$ receptors (Froestl and Mickel 1997). Although these chemical tools have been useful for studying the neurophysiology and molecular biology of GABA$_B$ receptors, they have failed for the most part to permit the identification of pharmacological subtypes. For example, using semi-intact tissue preparations it is possible to record robust pre- and postsynaptic responses to ba-

NEUROPSYCHOPHARMACOLOGY 2000–VOL. 23, NO. S4
© 2000 American College of Neuropsychopharmacology
Published by Elsevier Science Inc.
655 Avenue of the Americas, New York, NY 10010

0893-133X/00/$–see front matter
PII S0893-133X(00)00145-7

clofen and high-affinity antagonists block these with roughly equal potency (Bon and Galvan 1996; Pozza et al. 1999; Seabrook et al. 1990). Although measurements of neurotransmitter release have permitted some groups to report selective antagonism (Fassio et al. 1994; Bonanno et al. 1996, 1997; Teoh et al. 1996; but see also Baumann et al. 1990), there is no general consensus about pharmacologically defined receptor subtypes (Bowery 1997). This is perhaps surprising for a receptor system that is both widespread and otherwise well characterized.

The identification of a gene encoding GABA$_B$ receptors proved to be at least as challenging as the pharmacological search for subtypes. Several groups attempted to expression clone a receptor using *Xenopus* oocytes (Uezono et al. 1998), or to biochemically characterize a protein from brain, affinity purified using a monoclonal antibody (Nakayasu et al. 1993). The cloning breakthrough came with the development of the photo affinity radioligand, CGP71872. Using this as a marker, Kaupmann and co-workers (Kaupmann et al. 1997) identified a clone, GABA$_B$R1, predicted to encode a heptahelical protein composed of 844 amino acids. This clone shares many features of other members of the metabotropic glutamate and Ca^{++}-sensing receptor family including a large extracellular, N-terminal domain having homology to bacterial amino acid binding proteins, such as LIVBP (O'Hara et al. 1993). When expressed heterologously in cell lines, the antagonist pharmacology of the cloned receptor was shown to be very similar to that of native receptors. In contrast, the apparent affinity of agonist ligands was about 100-fold less than expected from studies of brain membranes. When challenged with an agonist, GABA$_B$R1 also did not appear to be able to stimulate the expected cellular responses, such as K$^+$ channel activation or robust inhibition of the accumulation of cAMP, which suggested that a necessary component of signal transduction was missing.

The publication of GABA$_B$R1 set off a search by many groups for other genes having a related sequence. A query of public databases using the GABA$_B$R1 sequence led to the identification of a second, homologous protein, GABA$_B$R2 (Jones et al. 1998; Kaupmann et al. 1998a; Kuner et al. 1999; White et al. 1998; Martin et al. 1999; Ng et al. 1999). Like GABA$_B$R1, GABAB$_B$R2 was found to contain a region homologous to LIVBP within the large N-terminal extracellular domain.

Several groups have shown that when expressed in either oocytes or mammalian cells GABA$_B$R2 fails to produce the anticipated cellular responses to GABA (Jones et al. 1998; Kaupmann et al. 1998a; White et al. 1998; but see also Kuner et al. 1999; Martin et al. 1999). This deficit in coupling was especially surprising considering the lack of function activity of GABA$_B$R1. Hinting at a resolution of the functional expression problem was the spatial distribution of the mRNAs for both GABA$_B$R1 and GABA$_B$R2. The *in situ* localization patterns of both

transcripts were highly convergent throughout the brain (Jones et al. 1998), suggesting that the two receptors were co-expressed in the same cellular regions. Double labeling of mRNAs and receptor proteins at cellular resolution provided proof that co-expression occurs in multiple cell types, including cerebellar Purkinje cells, hippocampal and cerebral cortical neurons, and the vast majority of neuronal somata within dorsal root ganglia (Jones et al. 1998; Kaupmann et al. 1998a; Durkin et al. 1999). We reasoned that fully functional GABA$_B$ receptors might somehow require the expression of both GABA$_B$R1 and GABA$_B$R2.

CO-EXPRESSION STUDIES

Co-expression of GABA$_B$R1 and GABA$_B$R2 in *Xenopus* oocytes and mammalian cells leads to the development of large amplitude GABA- and baclofen-sensitive GIRK currents (Jones et al. 1998; Kaupmann et al. 1998a; Kuner et al. 1999; White et al. 1998; Ng et al. 1999). These currents are rarely seen in cells expressing either gene alone (Kaupmann et al. 1998a, 1998b). Co-expression also permits robust inhibition of adenylyl cyclase (Kuner et al. 1999; Ng et al. 1999) as well as stimulation of GTPγS binding in cell membranes (White et al. 1998). The pharmacology of agonists at the GABA$_B$R1/R2 combination (Jones et al. 1998; Brauner-Osborne and Krogsgaard-Larsen 1999; Lingenhoehl et al. 1999) is comparable to that reported for native receptors (Bon and Galvan 1996; Seabrook et al. 1990). Antagonist affinity estimates for saclofen, CGP54626 and CGP55845 (Jones et al. 1998; Brauner-Osborne and Krogsgaard-Larsen 1999) are similar to values reported in previous electrophysiological studies using brain tissue (Bon and Galvan 1996; Seabrook et al. 1990), as well as to those obtained by measuring displacement of radioligand from cells expressing GABA$_B$R1 alone (Kaupmann et al. 1997).

Just as striking as the appearance of functional cellular responses is the shift in affinity for agonists. The co-expression of GABA$_B$R2 with GABA$_B$R1 now results in an apparent shift (10–30-fold) to a higher affinity state for the agonists GABA and baclofen (Kaupmann et al. 1998a; White et al. 1998). This observation led to the speculation that the two GABA$_B$ gene products might closely associate with one another (Kaupmann et al. 1998a). Additional evidence came from epitope tagging experiments that showed a high coincidence in the intracellular distribution of the two proteins when expressed heterologously (Jones et al. 1998).

PHYSICAL ASSOCIATION BETWEEN GABA$_B$R1 AND GABA$_B$R2

Using immunoprecipitation methods, several groups demonstrated that GABA$_B$R1 and GABA$_B$R2 specifically

associate in a protein complex, probably as heterodimers (Jones et al. 1998; Kaupmann et al. 1998a; Kuner et al. 1999; White et al. 1998). Furthermore, the heterodimers are concentrated on the plasma membrane, which suggests that the dimer is likely to be important for early events in signal transduction including ligand binding and G-protein activation (Jones et al. 1998). These studies provided the first compelling evidence that native G-protein-coupled receptors (GPCRs) can exist not only as homodimers, as is likely the case for other GPCRs (Gouldson and Reynolds 1997; Hebert and Bouvier 1998; Bai et al. 1998) but also as heterodimers composed of more than one receptor subunit.

Additional groups independently came to the conclusion that GABA_B receptors are formed of heterodimers, and in the process provided a molecular mechanism for the subunit interaction. Experiments with the yeast two-hybrid system for identifying protein partners led to the discovery of domains on the extended C-termini of GABA_BR1 and GABA_BR2 that are responsible for their molecular association (White et al. 1998; Kuner et al. 1999). Both C-termini are predicted to contain coiled-coil motifs based on algorithms that reliably detect such features in the primary amino acid sequences of other more well known proteins that dimerize (Lupas 1997; Lupas et al. 1991). When either of the coiled-coil structures are deleted, the proteins no longer associate (Kuner et al. 1999). The yeast two-hybrid experiments also revealed that the associations are strictly heterophillic; there is no evidence that homodimers form.

The coiled-coil structure provides the first molecular substrate for GPCR dimerization; but among GPCRs this structure is so far unique to GABA_B receptors (Table 1). In contrast, the coiled-coil is well conserved within GABA_B receptors from different species (Figure 1). Additional types of protein–protein interactions must be responsible for dimerization of other GPCRs. In the case of the metabotropic glutamate/Ca^{++} sensor family of GPCRs, cysteine disulfide bridges are at least partly responsible for dimer formation. It is very likely that interactions between transmembrane alpha helices are also critical for dimer formation or stabilization (Gouldson and Reynolds 1997; Hebert et al. 1996; Maggio et al. 1996; George et al. 1998).

CONSEQUENCES OF HETERODIMERIZATION

The heterodimer model immediately raises a myriad of questions about the roles of the two receptor proteins in signal transduction. Does only GABA_BR1 bind ligand? Do both subunits bind G-protein? Does GABA_BR2 merely serve as a shuttle protein, or does it have an active role signaling? Does any part of the signal transduction cascade involve dissociation of the two subunits?

Answers to some of these questions are beginning to emerge. For example, heterodimerization appears to be important for receptor trafficking to the plasma membrane. Several groups have noted that GABA_BR1, when expressed by itself, does not reach the plasma membrane; instead, it accumulates within the cytoplasm, probably in association with the endoplasmic reticulum (Couve et al. 1998; White et al. 1998). Even when over-expressed in neurons, which might be expected to correctly process and transport neuronal proteins, there is a deficit of plasma membrane labeling (Couve et al. 1998). Using fluorescence activated cell sorting and antibodies that recognize an extracellular epitope of GABA_BR1b, White et al. (1998) observed that the inclusion of GABA_BR2 induces a significant increase in the surface expression of GABA_BR1b, and presumably, the heterodimer. In the absence of GABA_BR2, GABA_BR1 also exhibits an immature pattern of glycosylation (White et al. 1998). Thus, GABA_BR2 has an important role in regulating the expression of the mature, signaling heterodimer. In this regard, the assembly and transport of GABA_B receptors may be similar to that of other proteins having multiple subunits, such as ion channels (Yu and Hall 1991). It is interesting to note that in a subset of neurons, such as hippocampal CA1 pyramidal cells and inhibitory interneurons,

**Table 1.** Analysis of GPCR Databases Using the COILS[a] Algorithm

GPCR Family	No. in Database[b]	Hits[c]	Description[d]
Rhodopsin	432	4	d1dr_fugru, m3dr_human, msr_xenla, opsd_octdo
Olfactory	491	0	
Secretin	29	0	
Chemosensor	8	0	
Frizzled	7	0	
mGluR/CaSR/GABA_B	32	7	GABA_BR1 (hum, rat, drosophila), GABA_BR2 (hum, rat, sponge, nematode)
Total	999	11	

[a] Lupas (1997).

[b] Search set includes GPCRs from all species in Genbank as of November 1999.

[c] Hits include those which show a >90% probability using a window size of 28 and weighting the first and fourth positions in the heptad repeat. Note that hits in the rhodopsin family do not extend to species homologues. Therefore, the significance for these receptors is less certain.

[d] Swiss Protein database nomenclature (except for GABA_B receptors).
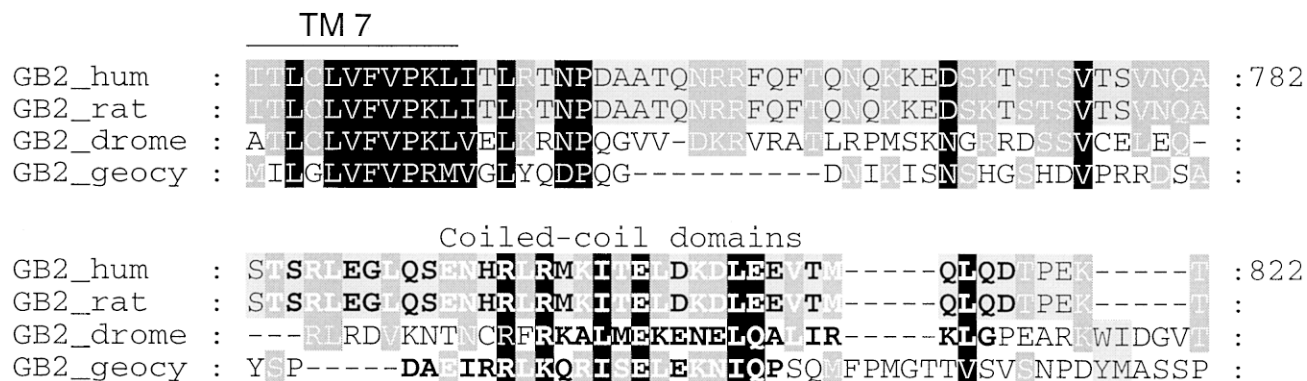
Figure 1.   Alignment of portions of the C-terminal regions of GABA$_B$R2 from four species. Alignment was made using the default settings of Clustal W. Shading shows different levels of amino acid conservation. Residues in bold comprise the coiled-coil domain as predicted using the algorithm "COILS" (Lupas 1997). GB2_drome, GABA$_B$R2 from Drosophila melanogaster; GB2_geocy, Geodia cydonium (a marine sponge).

GABA$_B$R1-immunoreactive protein appears abundantly in the cytoplasm (Sloviter et al. 1999). Although the expression levels of GABA$_B$R2 protein have not been published for these cells, the CA1 cells do exhibit a much lower level of mRNA encoding this protein as compared to cells in nearby CA3 (Durkin et al. 1999; Kuner et al. 1999). These observations lead to the speculation that GABA$_B$R2 protein may be constitutively low in certain cell types resulting in an inefficient transport of GABA$_B$R1 to distal plasma membrane surfaces (Sloviter et al. 1999). The actual extent to which GABA$_B$R2 protein regulates expression of the functional heterodimer remains to be determined.

Immunochemical methods have demonstrated that native GABA$_B$ receptors in the nervous system exist largely as heterodimers. Throughout the CNS there is a striking degree of overlap of GABA$_B$R1 and GABA$_B$R2 immunoreactivity at a gross structural level (Benke et al. 1999). In the cerebellar molecular layer co-localization of GABA$_B$R1 and GABA$_B$R2 immunoreactivity has been observed in ultra-thin sections of Purkinje cell spines (Kaupmann et al. 1998a) providing support for co-assembly at particular postsynaptic structures. Since GABA$_B$ receptors are so widespread in the CNS, a useful approach to estimate the overall proportion of receptors that are composed of heterodimers versus homodimers is to perform quantitative immunoprecipitations on fractions containing solubilized receptors. Using this method, Benke and colleagues (Benke et al. 1999) observe that essentially all GABA$_B$R1 protein is immunoprecipitated with antibodies that recognize GABA$_B$R2. Thus, the proportion of receptors in the brain that exist either as GABA$_B$R1 or GABA$_B$R2 monomers is thought to be quite low.

FUNCTIONS OF THE HETERODIMER SUBUNITS

High-affinity agonists and antagonists bind the GABA$_B$R1 subunit in a region which exhibits structural homology to the ligand-binding domain of metabotropic glutamate receptors and bacterial periplasmic amino acid binding proteins (Galvez et al. 1999). And, since the antagonists are capable of completely blocking cellular responses to GABA or baclofen, it is clear that the ligand-binding domain on the N-terminus of GABA$_B$R1 is essential for initiating signal transduction by the heterodimer. GABA$_B$R2 does not bind radiolabeled GABA$_B$R1 antagonists, nor does it appear to bind [^{3}H]-GABA (Jones et al. 1998; Kaupmann et al. 1998; White et al. 1998). There have, however, been reports that GABA$_B$R2, when expressed alone, can be stimulated by GABA (Kaupmann et al. 1998a; Kuner et al. 1999; Martin et al. 1999). Does GABA$_B$R2 contribute a binding site for GABA or another ligand? The first possibility explored was sensitivity to Ca^{++} since GABA$_B$R2 exhibits some sequence similarity to the Ca^{++}-sensing receptor (Ruat et al. 1995). In fact, Ca^{++} can be seen to strongly modify the response of the heterodimer to GABA, but this effect can be attributed entirely to specific amino acid residues within the ligand-binding region of GABA$_B$R1 (Wise et al. 1999; Galvez et al. 2000).

To explore a ligand-binding role for GABA$_B$R2, we performed mutations within the region of GABA$_B$R1b that encodes the ligand-binding domain, and then assessed the functional status of the heterodimer by expression with GABA$_B$R2. The mutations that were performed are described in Figure 2. Using [3H]-CGP54626 as a radiolabel, specific binding was reduced by more than 85% in membranes expressing any of the GABA$_B$R1b mutants (data not shown). Others have shown that the S246A and Y266A mutations cause a >1000-fold decrease in affinity for antagonists using a different radioligand (Galvez et al. 1999). To determine if the mutations created a parallel decrease in functional activity, receptors were assayed for Ca^{++}-mobilization in a fluorescence plate reader using Fluo-3 dye and a chimeric G-protein (Conklin et al. 1993), G$\alpha_{q/i3(5)}$, that permits coupling of the heterodimer to activation of phospholipase C. Co-expression of non-

A

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                    246                                266 269
    GBR1b   ..ILMPGCSSVSTLVAEAARMWNLIVLSYGSSSPALSNR..
    mGluR1  ..VIGPGSSSVAIQVQNLLQLFDIPQIAYSATSIDLSDK..
    LIVPB   ..VIGHLCSSSTQPASDIYEDEGILMISPGATAPELTQR..
    CSR1a   ..VVGATGSGVSTAVANLLGLFYIPQVSYASSSRLLSNK..
    GBR2    ..MVFGGVCPSVTSIIAESLQGWNLVQLSFAATTPVLAD..
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B

GABA$_B$R1b

GABA$_B$R2   *Bgl*II

Figure 2. **(A)** Alignment of a portion of the ligand-binding domains of GABA$_B$R1b (GBR1b), GABA$_B$R2 (GBR2), mGluR1, Ca^{++} sensor (CSR1a), and LIVBP. Bold letters in GABA$_B$R1 indicate residues that were mutated in this study. **(B)** Schematic of GABA$_B$R1 and GABA$_B$R2 showing the approximate locations of the region shown in detail in (A) (diagonals) and the *Bgl*II fragment (stippled region) deleted from GABA$_B$R2. *Bgl*II digestion of plasmid containing the GABA$_B$R2 cDNA removes 771 nucleotides encoding amino acids 226–482 in the protein. Signal peptide sequence (vertical stripes) and transmembrane regions (gray boxes) are indicated. All mutant and deletion constructs were verified by sequence analysis.

mutated GABA$_B$R1b with GABA$_B$R2 resulted in concentration-dependent increases in liberated Ca^{++} with GABA and baclofen. EC$_{50}$ values for these agonists were similar to those reported using other methods. In contrast, co-expression of either S246A or Y266A mutants with wild-type GABA$_B$R2 failed to stimulate Ca^{++} release using concentrations of GABA or baclofen up to 1000-fold above their EC$_{50}$ values (Figure 3). These mutant receptors appeared to be expressed based on the appearance of cellular immunofluorescence using antibodies directed against the epitope-tagged C-terminus (data not shown). Thus, the most likely explanation for the lack of functional activity even at 1000-fold higher concentrations of agonist is that activation of the heterodimer is absolutely dependent on agonist binding to the GABA$_B$R1b subunit. Others have shown that the S269A substitution induces a selective reduction in affinity to GABA while sparing baclofen binding (Galvez et al. 1999). When expressed with GABA$_B$R2, a similar shift in the EC$_{50}$ of GABA, but not baclofen, was observed for the Ca^{++} mobilization response (Figure 3). The parallel changes in agonist potency measured either as binding to GABA$_B$R1b, or as activation of the heterodimer, provides additional support for the view that GABA$_B$R2 cannot directly contribute to receptor activation by either of these ligands.

The coiled-coil domain on the C-terminus of GABA$_B$R2 is necessary for binding GABA$_B$R1 and for formation of functional heterodimers (Kuner et al. 1999; White et al. 1998). Protein assemblies mediated by coiled-coil domains are generally thought to be thermodynamically quite stable. This and the observed predominance of GABA$_B$ receptor heterodimers over monomers (Benke et al. 1999) strongly suggests that the GABA$_B$ receptor is likely to remain as a heterodimer throughout the cycle of agonist binding, receptor activation and G-protein stimulation. What direct role, if any, does GABA$_B$R2 have in any of these events? The lack of conservation of the agonist-

A

B
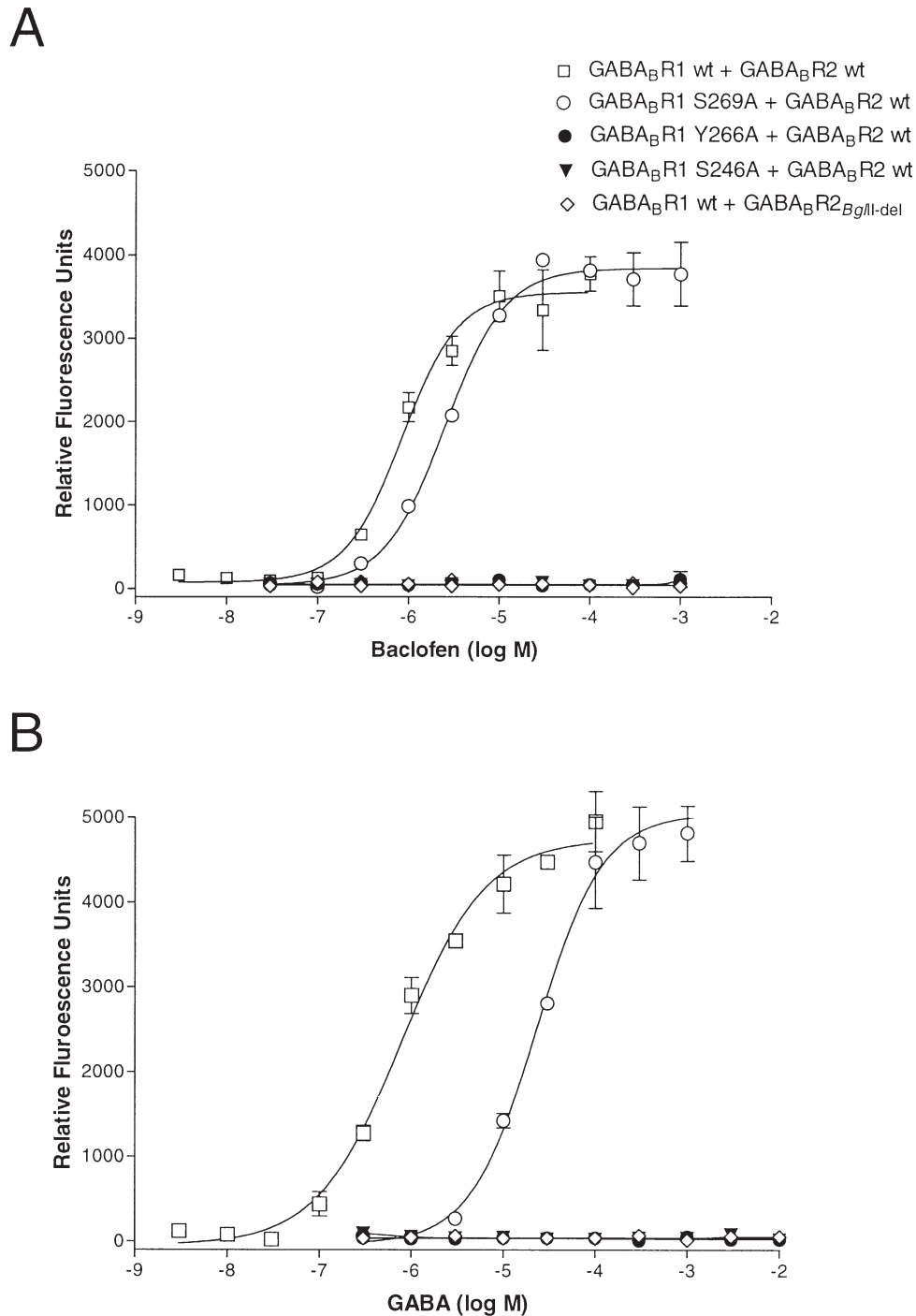
Figure 3. Activation of wild-type and mutant receptors by GABA and baclofen. Intracellular Ca^{++} release was measured by Fluo-3 dye fluorescence using a FLIPR (molecular devices). Cells were transiently transfected with plasmids containing wild-type or mutant $GABA_BR1b$ and wild-type or truncated $GABA_BR2$ and chimeric $G\alpha_{q/i3(5)}$. Data are expressed as means ± S.E.M. of a quadruplicate determination from a single experiment. Similar results were obtained in three separate experiments.

binding domain of $GABA_BR1$ in $GABA_BR2$ would indicate that point mutations might not be an efficient way to map out domains important for signaling by $GABA_BR2$.

As a first step toward determining domains on $GABA_BR2$ important for signal transduction, we performed a deletion of 257 amino acids within the N-ter-minal domain of $GABA_BR2$ (Figure 2B). The expression pattern of this deletion mutant ($GABA_BR2_{BglII}$-del) was similar to that of wild-type $GABA_BR2$ as judged by the appearance of cellular immunofluorescence using anti-bodies directed against the epitope-tagged C-terminus (data not shown). In COS-7 cells co-expressing $GABA_BR1b$

and GABA$_B$R2$_{Bgl}$II-del no evidence was found for receptor activation by either GABA or baclofen using the Ca^{++}-mobilization assay (Figure 3). Loss of this region within the N-terminus of GABA$_B$R2$_{Bgl}$II-del may prevent heterodimerization, or it may produce a more subtle change in structure of the heterodimer that prevents stimulation of G-protein.

SUMMARY

GABA$_B$ receptors may be thought of as being composed of two subunits, a ligand-binding subunit, GABA$_B$R1, and a "structural" subunit, GABA$_B$R2. GABA$_B$R1 appears to determine the pharmacological properties of the receptor, while GABA$_B$R2 is important for proper expression of GABA$_B$R1 on the plasma membrane. Additional deletion and mutagenesis experiments will be required to determine to what extent GABA$_B$R2 actually participates in signal transduction events.

Suddenly it appears that the field of G-protein-coupled receptors is playing catch-up with its brethren plasma membrane proteins: ion channels, receptor tyrosine kinases and transporters, most of which occur as oligomeric assemblies. GABA$_B$ receptors are now joined by a growing list of GPCRs which are thought to exist as heterodimers, including receptors for endogenous opiates (Jordan and Devi 1999), dopamine and somatostatin (Rocheville et al. 2000) and serotonin (Xie et al. 1999). The recent finding that heterodimerization can change receptor affinities for agonists (Jordan and Devi 1999) indicates that the heteromeric nature of GPCRs is likely to have major implications for drug design in the near future. Given the fact that heterodimerization is now seen to occur in two divergent families of GPCRs, it seems likely that additional examples will become known with further study.

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