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$GABA_{B2}$ Is Essential for G-Protein Coupling of the $GABA_B$ Receptor Heterodimer

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$GABA_B$ receptors are unique among G-protein-coupled receptors (GPCRs) in their requirement for heterodimerization between two homologous subunits, $GABA_{B1}$ and $GABA_{B2}$, for functional expression. Whereas $GABA_{B1}$ is capable of binding receptor agonists and antagonists, the role of each $GABA_B$ subunit in receptor signaling is unknown. Here we identified amino acid residues within the second intracellular domain of $GABA_{B2}$ that are critical for the coupling of $GABA_B$ receptor heterodimers to their downstream effector systems. Our results provide strong evidence for a functional role of the $GABA_{B2}$ subunit in G-protein coupling of the $GABA_B$ receptor heterodimer. In addition, they provide evidence for a novel "sequential" GPCR signaling mechanism in which ligand binding to one heterodimer subunit can induce signal transduction through the second partner of a heteromeric complex.

Key words: $GABA_B$; GPCR; dimerization; signaling; G-protein coupling; receptor subunits

$GABA_B$ receptors are G-protein-coupled receptors (GPCRs) that mediate slow synaptic inhibition in the brain and spinal cord (for review, see Bowery, 1993; Kerr and Ong, 1995; Couve et al., 2000). They are unique among type C GPCRs in that they are heterodimers of $GABA_{B1}$ (Kaupmann et al., 1997) and $GABA_{B2}$ subunits (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999), and it is now generally accepted that each subunit is unable to form a functional receptor when expressed in isolation (Kaupmann et al., 1997; Jones et al., 1998; White et al., 1998; Ng et al., 1999). With respect to $GABA_{B1}$, this is attributable to its retention within the endoplasmic reticulum on homomeric expression (Couve et al., 1998; Calver et al., 2000; Filippov et al., 2000; Margeta-Mitrovic et al., 2000). In this context, $GABA_{B2}$ has been shown to play a key role in trafficking the $GABA_{B1}$ subunit to the cell surface (Couve et al., 1998; Filippov et al., 2000). This exposes the N-terminal ligand binding domain of $GABA_{B1}$, which is not present in $GABA_{B2}$ (Galvez et al., 2000a,b), enabling it to bind extracellular agonist and subsequently activate downstream signaling pathways. Interestingly, it has been shown recently that C-terminal $GABA_{B1}$ mutants, which are expressed on the cell surface in the absence of $GABA_{B2}$, are completely nonfunctional, despite their ability to bind GABA (Calver et al., 2000; Margeta-Mitrovic et al., 2000). $GABA_B$ receptor function is restored, however, when these mutants are coexpressed with $GABA_{B2}$, demonstrating that the $GABA_{B2}$ subunit may not only be important for correct trafficking of $GABA_{B1}$ but also for the mediation of agonist-induced G-protein coupling. This hypothesis has been given additional strength by a recent report by Galvez et al. (2001), demonstrating that the extracellular domain of $GABA_{B2}$ is essential for agonist activation of the heterodimeric receptor.

Mechanisms for G-protein coupling have been investigated widely for members of the rhodopsin–β-adrenergic GPCR family. Growing evidence suggests that the second and third intracellular loops are critical interaction sites important for both G-protein coupling and selectivity. In addition, these intracellular domains have been demonstrated to be important for G-protein coupling of type C GPCRs, such as metabotropic glutamate receptors (mGluRs) (Gomeza et al., 1996; Francesconi and Duvoisin, 1998). At present, the role of the two $GABA_B$ subunits in G-protein coupling and downstream signal transduction is unknown. Here, using a site-directed mutagenesis approach, we investigated the importance of residues in the second intracellular loop (il2) of $GABA_{B1}$ and $GABA_{B2}$ subunits. We demonstrated that mutations within il2 of $GABA_{B2}$ can dramatically decrease responses of the heterodimer complex to agonist, to the extent that receptor signaling can be effectively abolished by the introduction of three negatively charged residues into this region. In contrast, reciprocal mutations made in il2 of the $GABA_{B1}$ subunit have no apparent effect on heterodimer signaling. These results are consistent with a model in which agonists bind to the $GABA_{B1}$ subunit, resulting in signal transduction via G-protein coupling through the $GABA_{B2}$ subunit. This would therefore suggest the existence of a novel "sequential and sideways" signaling cascade that may be of relevance to the growing number of reported GPCR heterodimers (Milligan and Rees, 2000).

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MATERIALS AND METHODS

Site-directed mutagenesis. The $GABA_{B1b}$ and $GABA_{B2}$ subunits were tagged with either an N-terminal c-myc (wt$GABA_{B1}$) or hemaglutinin (HA) (wt$GABA_{B2}$) epitope tag (Calver et al., 2001). PCR primers were designed to include single, double, triple, or quintuple amino acid changes in both the wt$GABA_{B1}$ and wt$GABA_{B2}$, resulting in 12 mutant constructs: $GABA_{B1}{}^{E579K}$, $GABA_{B1}{}^{E583K}$, $GABA_{B1}{}^{E579K/E580K}$, $GABA_{B1}{}^{E579K/E580K/E583K}$ (referred to as $GABA_{B1}{}^{tripleK}$), $GABA_{B2}{}^{K586E}$, $GABA_{B2}{}^{K590E}$, $GABA_{B2}{}^{K586E/M587E,}$ $GABA_{B2}{}^{K586E/M587E/K590E}$ (referred to as $GABA_{B2}{}^{tripleE}$), $GABA_{B1}{}^{K577/578A}$, $GABA_{B1}{}^{K581/582A}$, $GABA_{B1}{}^{K577/578/581/582/586A}$ (referred to as $GABA_{B1}{}^{KQA}$), and $GABA_{B1}{}^{K577/578/581/582/586E}$ (referred to as $GABA_{B1}{}^{KQE}$). Mutagenesis experiments were performed using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer, and all amino acid changes were confirmed by full double-stranded sequencing.

C-terminal truncates (from amino acid 747 of $GABA_{B1}$) of the $GABA_{B1}$ mutant constructs (glutamate to lysine only) were made by PCR amplification using forward primer 5′-CCCGAATTCATGGG GCCCGGGGCC-3′ and reverse primer 5′-CCCAAGCTTCCTCG GGTGATCAGCCTG-3′. A $GABA_{B1N/2}$ chimera was generated by ligation of two PCR products ($GABA_{B1b}$ 1–470 and $GABA_{B2}$ 479–954). PCR amplification was performed using forward primer 5′-CCCGA ATTCATGGGGCCCGGGGCC-3′ and reverse primer 5′-CCCAAGC TTCTGGTCAGCTGGGGGGGAC-3′ to generate fragment $GABA_{B1b}$ 1–470. PCR amplification with forward primer 5′-CCCAAGCTT ATCATCCTGG AGCAGCTGCGGAAG-3′ and reverse primer 5′-CCCCTCGAGTTACAGGCCCGAGACCA TGACTC-3′ was used to generate fragment $GABA_{B2}$479–954. All constructs were cloned into the eukaryotic expression construct pcDNA3.1 (Invitrogen, Paisley, UK). Schematic representations of these constructs are shown in Figure 1*c*.

Transfection. Human embryonic kidney 293 (HEK293) cells were transfected with either wild-type (wt) or mutant $GABA_{B1}$- or $GABA_{B2}$-containing plasmids using Lipofectamine Plus (Life Technologies, Paisley, UK) according to the instructions of the manufacturer. Cells were maintained in Minimal Essential Medium supplemented with 10% fetal bovine serum and 1% nonessential amino acids (all from Life Technologies). After transfection, cells were left for 24 hr before being subcultured for immunocytochemistry or the Ca^{2+} mobilization assay.

The $GABA_{B1b}$/$GABA_{B2}$ cell line was generated in Chinese hamster ovary cells as described previously (Hirst et al., 2000). The $GABA_{B2}$ cell line was generated in HEK293 cells by transient transfection of the $GABA_{B2}$ cDNA as described above and selection of positive cells in 800 μg/ml G418.

Immunocytochemistry. HEK293 cells transfected with either wild-type or mutant constructs were subcultured onto glass coverslips, fixed with 4% paraformaldehyde, and then either permeabilized with 0.1% Triton X-100 for 10 min or washed with PBS. Cells were incubated with primary antibody [anti-c-myc, 1:5000; anti-HA, 1:5000 (Boehringer Mannheim, Brüssel, Belgium); and anti-$GABA_{B1b}$, 1:1000] for 60 min and washed, and then secondary antibody [goat anti-mouse IgG FITC for c-myc; goat anti-rat IgG FITC for HA (Sigma, St. Louis, MO); and goat ant-rabbit IgG FITC for $GABA_{B1b}$] was used at 1:100 dilution for 60 min. Cells were finally washed, mounted onto glass slides using Citifluor (Citifluor Ltd., London, UK), and viewed using a Leica (Nussloch, Germany) confocal microscope.

Ca^{2+} mobilization assay. Transfected cells were seeded into 96-well plates at a density of 50,000 cells per well and incubated at 37°C in 5% CO_2 for 24 hr before use. The intracellular Ca^{2+} mobilization in response to agonist was then measured as described previously (Calver et al., 2000). The data were iteratively curve fitted using a four parameter logistic model (Bowen and Jerman, 1995) as mean ± SEM (with each data point being determined in triplicate) of one representative experiment. Significance of data were assessed using a one-way ANOVA, followed by *post hoc t* test (least square difference). Data were considered significant if $p < 0.05$.

Radioligand binding. Membranes from transfected cells were prepared, and radioligand binding to [^{3}H]CGP54626 was performed as described previously (Calver et al., 2000). The concentration of drug-inhibiting-specific radioligand binding by 50% (IC_{50}) was determined by iterative curve fitting (Wood et al., 2000). pK_i values (the negative $\log_{10}$ of the molar K_i) for receptor binding were then calculated from the IC_{50} values as described by Cheng and Prusoff (1973) using K_D values determined previously in saturation binding studies (3 nM). B_{max} values were calculated from the specific bound accounting for receptor occupancy at this radioligand concentration using the Hill–Langmuir adsorption isotherm. Data are expressed as the mean ± SEM of at least three separate experiments from two independent sets of transfections.

[^{35}S]GTPγS binding assays. Cells were homogenized in 20 mM HEPES and 10 mM EDTA, pH 7.4 (4°C), and centrifuged at 48,000 × *g* for 20 min at 4°C. Membrane pellets were rehomogenized in 20 mM HEPES and 0.1 mM EDTA, pH 7.4, (4°C). The pellet was recentrifuged as described above, the supernatant was discarded, and the pellet was resuspended in 20 mM HEPES and 0.1 mM EDTA and stored at −80°C until required.

[^{35}S]GTPγS binding assays were performed in a 20 mM HEPES buffer,

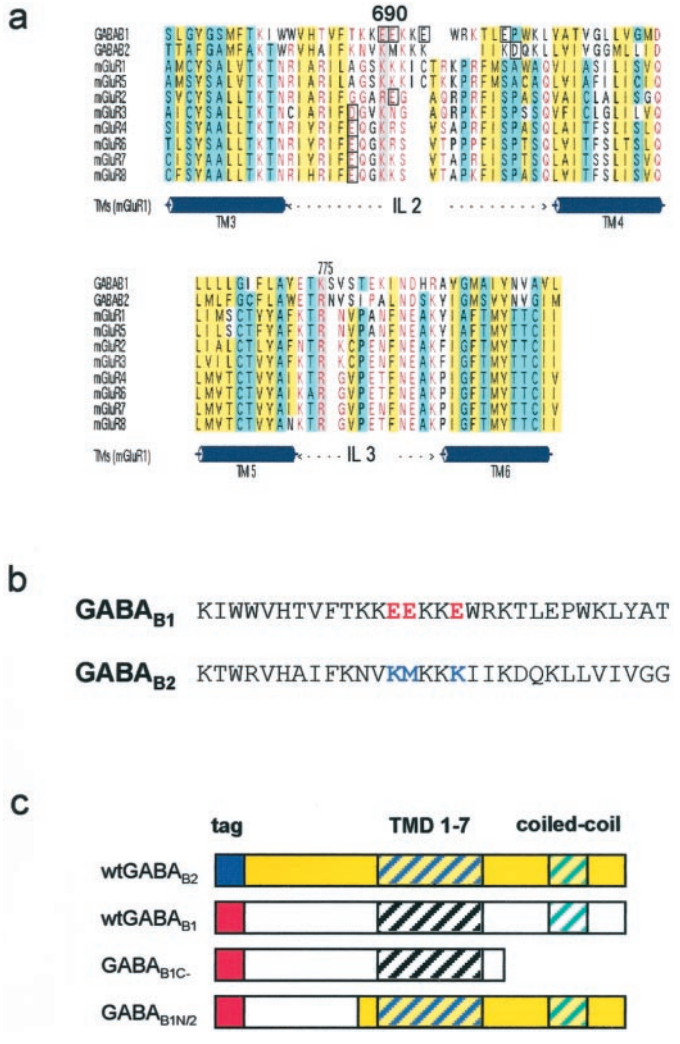

Figure 1. Intracellular loop sequence alignments and summary of $GABA_{B1}$ and $GABA_{B2}$ constructs. *a*, Alignment of the second and third intracellular loops of $GABA_{B1}$ (*GABAB1*) and $GABA_{B2}$ (*GABAB2*) subunits with members of the mGluR family. The alignment was produced in Alscript using HMMALIGN and the G-protein-coupled receptors database (GPCRDB) (Barton, 1993; Horn et al., 1998). The *shading* indicates different levels of amino acid conservation, in which residues are colored if eight or more residues show conservation of amino acid properties: *blue shading*, small; *yellow shading*, hydrophobic; *red characters*, polar. Transmembrane helices (*TMs*, *dark blue*) are displayed for mGluR1 (as assigned in SwissProt, entry MGR1 HUMAN). *Boxed letters* indicate residues that are negatively charged. Conserved residues discussed in Results are shaded in *gray*. The alignment has been extracted from a larger alignment of family 3 GPCRs taken from GPCRDB (Horn et al., 1998). *b*, Enlarged alignment of the second intracellular loop of both $GABA_{B1}$ and $GABA_{B2}$ subunits. *Bold red letters* indicate residues mutated in this study. *c*, $GABA_B$ receptor subunit truncations and chimeras. Epitope tags are marked as *red* (c-myc) or *blue* (HA) *boxes*. Transmembrane domains 1–7 (*TMD 1–7*) are represented by *black striped boxes.*. The coiled-coil domains are depicted by *green striped boxes*.

pH 7.4, containing 3 mM $MgCl_2$ and 100 mM NaCl. Cell membranes were preincubated with 10 μM GDP, and increasing concentrations of GABA or baclofen for 30 min at 30°C and then 0.1 nM [^{35}S]GTPγS was added to each well and incubated for an additional 30 min. Nonspecific binding was determined in the presence of 20 μM GTPγS. The incubation was terminated by rapid filtration through J. Whatman glass fiber filters (GF/B) (Semat International, St. Albans, UK) and washed with 3 ml of ice-cold HEPES buffer, pH 7.4, containing 3 mM $MgCl_2$. Radioactivity was determined by liquid scintillation spectrometry using a Packard (Meridian, CT) TopCount.

cAMP accumulation assays. cAMP levels in cells were determined by radioimmunoasay (SMP004; NEN, Boston, MA) according to the instructions of the manufacturer. In brief, cells were washed once with Ca^{2+}-free PBS, scrapped up in the same buffer, and pelleted by a 5 min centrifugation at 400 × *g*. The pellet resuspended in manufacturers stimulation buffer, and ~50,000 cells were added to the appropriate wells of the NEN flash plates together with 10 μM forskolin, plus increasing concentration of agonist. Plates were incubated for 15 min at 37°C, before the addition of the manufacturers detection mixture containing [^{125}I]cAMP tracer (0.16 μCi/ml) to the wells. Plates were covered and left for 24 hr before counting on a Packard TopCount.

Neuron preparation and cDNA injection. Neuron isolation and injection procedures have been described previously (Caulfield et al., 1994; Couve et al., 1998; Filippov et al., 2000). Briefly, single superior cervical ganglion (SCG) neurons were dissociated from 15-to 19-d-old rats and plated on laminin-coated glass coverslips. Five hours after plating, neurons were microinjected into the nucleus with plasmids carrying cDNAs for the $GABA_B$ receptor subunits, in addition to a plasmid encoding green fluorescent protein to subsequently identify successfully injected cells. Electrophysiological recordings were routinely made 16–20 hr after injection at room temperature (20°C).

Ca^{2+} channel current recording. Currents through voltage-gated Ca^{2+} channels were recorded using the conventional whole-cell patch-clamp method as described previously (Caulfield et al., 1994; Couve et al., 1998; Filippov et al., 2000). Ca^{2+} channel currents were routinely evoked every 20 sec with 100 msec depolarizing rectangular test pulse to 0 mV from a holding potential of −90 mV. Ca^{2+} channel current amplitudes were measured isochronally 10 msec from the onset of the rectangular test pulse, i.e., near to the peak of the control current. As reported previously (Filippov et al., 2000), currents were primarily N-type with negligible contribution by L-type channels. A racemic mixture of (+)- and (−)-baclofen was used in all experiments. Data are presented as means ± SEM as appropriate. Student's test (unpaired) was applied to determine statistical significance. Difference were considered significant if $p < 0.05$.

RESULTS

Sequence analysis of the second and third intracellular loops of $GABA_{B1}$ and $GABA_{B2}$

Using sequence alignments, we compared residues in the second and third intracellular loops of the $GABA_B$ subunits and the related type C mGluRs (Fig. 1*a*). The basic residue Arg^{775} in the third intracellular loop (il3) of mGluR1 has been shown to be important for coupling to G-proteins (Francesconi and Duvoisin, 1998) and is conserved throughout the mGluR family and also in $GABA_{B2}$ (Fig. 1*a*). In contrast, the corresponding residue in $GABA_{B1}$ is a lysine. In il2 of all of the mGluRs, there is a conserved lysine at position 690 (numbered with respect to mGluR1), except for mGluR2 in which the equivalent residue is an arginine, and this basic residue has been shown to be crucial for the interaction of the mGluRs with their respective G-protein (Francesconi and Duvoisin, 1998). Similarly, we noted that $GABA_{B2}$ also has a basic lysine at this key position. This is in contrast to the $GABA_{B1}$ subunit, in which the acidic amino acid glutamate is substituted for this lysine, resulting in a reversal of charge at this site. These findings may be of functional relevance, because it has been proposed previously that electrostatic interactions between positive charges in the intracellular domains of GPCRs may be important for coupling to a negatively charged face of the G-protein (Fanelli et al., 1998).

Amino acids in the second intracellular loop of $GABA_{B2}$ are critical for $GABA_B$ receptor signaling through the chimeric G-protein G_{qi5}

Based on our sequence analysis of the $GABA_{B1}$ and $GABA_{B2}$ intracellular loops, we targeted three amino acid residues (K586, M587, and K590) within il2 of $GABA_{B2}$ for mutagenesis studies (Fig. 1*a*,*b*). These residues were mutated to glutamates either individually or in combination to give a series of single, double, or triple mutants: $GABA_{B2}{}^{K586E}$, $GABA_{B2}{}^{K590E}$, $GABA_{B2}{}^{K586E/M587E}$, and $GABA_{B2}{}^{tripleE}$ (Fig. 2*a*). When transfected into HEK293 cells in isolation, both wild-type and mutant $GABA_{B2}$ subunits could be detected on the cell surface in the absence of membrane permeabilization (Fig. 2*b*). In addition, coexpression of each $GABA_{B2}$ mutant with wt$GABA_{B1}$ demonstrated that each mutant retained its ability to traffic the $GABA_{B1}$ subunit to the cell surface (Fig. 2*b*). Having demonstrated that these mutants behaved normally in terms of trafficking to the cell surface, we next tested the ability of the mutated $GABA_{B2}$ subunits to functionally couple to G-proteins when coexpressed with the wild-type $GABA_{B1}$ subunit. Although $GABA_B$ receptors are known to inhibit adenylyl cyclase activity by preferentially coupling to $G_{o/i}$, they can also be made to signal via the phospholipase C pathway when coexpressed with the chimeric G-protein G_{qi5} (Franek et al., 1999; Wood et al., 2000). Activation of this pathway results in a mobilization of intracellular Ca^{2+} stores, which can then be measured on a fluorimetric imaging plate reader (FLIPR). Coexpression of wt$GABA_{B1}$ and wt$GABA_{B2}$ subunits with G_{qi5} in HEK293 cells resulted in a robust Ca^{2+} mobilization in response to GABA (pEC_{50}, 7.19 ± 0.06; $n = 36$). In the same system, expression of wt$GABA_{B1}$ with either $GABA_{B2}{}^{K586E}$ or $GABA_{B2}{}^{K590E}$ resulted in significantly reduced pEC_{50} values when compared with coexpression with wt$GABA_{B2}$ (6.35 ± 0.07, $n = 11$, $p < 0.001$; and 6.07 ± 0.04, $n = 12$, $p < 0.001$, respectively) (Fig. 2*c*). Expression of wt$GABA_{B1}$ with the double-mutant $GABA_{B2}{}^{K586E/M587E}$ resulted in additional reduction of the observed pEC_{50} in response to GABA (5.40 ± 0.11; $n = 10$; $p < 0.001$). In contrast to the other $GABA_{B2}$ mutants studied, expression of $GABA_{B2}{}^{tripleE}$ in combination with wt$GABA_{B1}$ resulted in no detectable responses in this functional assay ($n = 12$) (Fig. 2*c*).

Based on these results, we next prepared mutations in the second intracellular loop of $GABA_{B2}$ that would help us investigate the relative importance of losing or gaining positive charge within this stretch of five amino acids. To do this, we examined the effects of changing residues K586, M587, and K590 to neutral alanines instead of negatively charged glutamates. As with the previous set of glutamate mutants, cell surface expression of each of the alanine mutants in isolation appeared no different to that of the wt$GABA_{B2}$ subunit (data not shown). Expression of wt$GABA_{B1}$ and G_{qi5} in combination with $GABA_{B2}{}^{K586A}$ or $GABA_{B2}{}^{K590A}$ resulted in significantly reduced pEC_{50} values when compared with coexpression with wt$GABA_{B2}$ (6.30 ± 0.13, $n = 8$, $p < 0.001$; and 5.75 ± 0.08, $n = 10$, $p < 0.001$, respectively) (Fig. 2*d*). Coexpression with the double-mutant $GABA_{B2}{}^{K586A/M587A}$ resulted in similar significant reduction in pEC_{50} in response to GABA (6.16 ± 0.07; $n = 8$; $p < 0.001$). In contrast to the other $GABA_{B2}$ mutants studied, and similar to the $GABA_{B2}{}^{tripleE}$ mutant, expression of $GABA_{B2}{}^{tripleA}$ in combination with wt$GABA_{B1}$ resulted in no detectable responses in this functional assay (Fig. 2*d*).

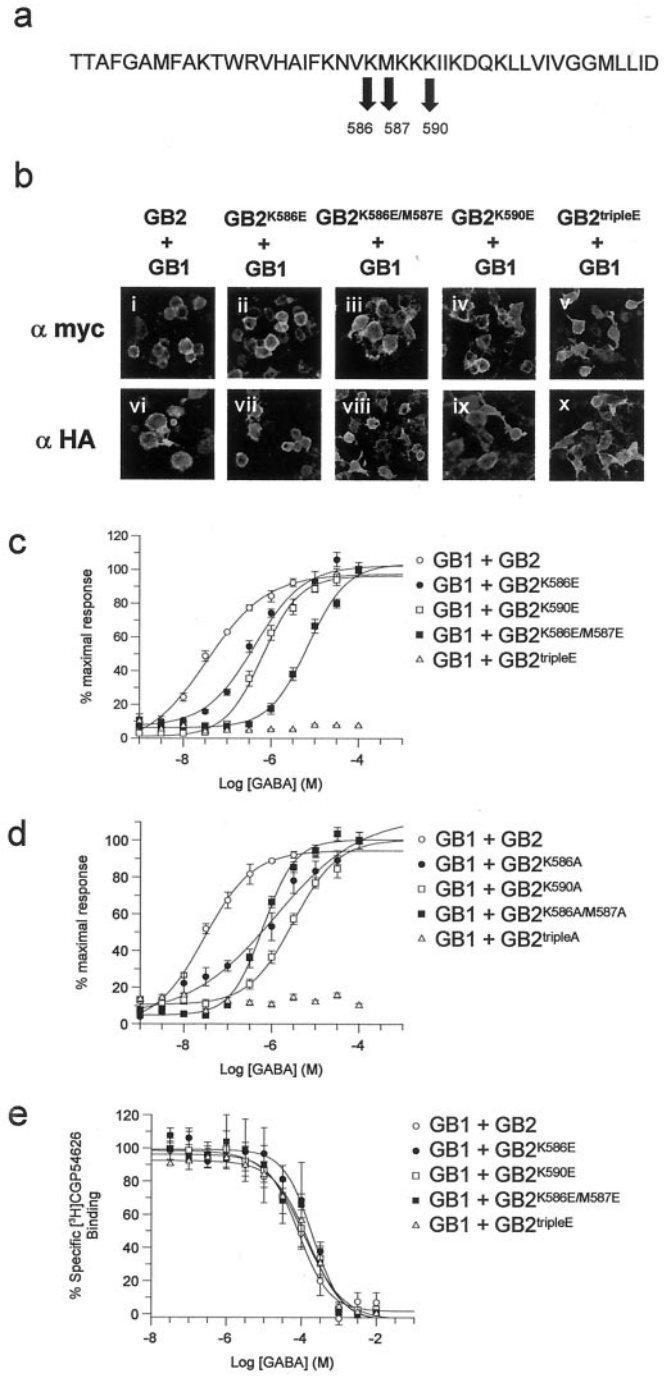

Figure 2. Residues within the second intracellular loop of $GABA_{B2}$ are critical for agonist-induced $GABA_B$ receptor signaling. *a*, Schematic representation of $GABA_{B2}$ il2. *Arrows* indicate residues mutated. *b*, HEK293 cells were transiently transfected with wt$GABA_{B1}$ (*GB1*) and wt$GABA_{B2}$ (*GB2*), $GB2^{K586E}$, $GB2^{K590E}$, $GB2^{K586E/M587E}$, or $GB2^{tripleE}$. Cell surface expression of $GABA_B$ subunits was examined by immunofluorescence using an anti-myc antibody for $GABA_{B1}$ (*i–v*) and an anti-HA antibody for $GABA_{B2}$ (*vi–x*). *c*, Representative FLIPR analysis of intracellular Ca^{2+} changes after GABA stimulation of cells transiently transfected with G_{qi5}, wt$GABA_{B1}$ (*GB1*), and wt$GABA_{B2}$ (*GB2*) $GB2^{K586E}$, $GB2^{K590E}$, $GB2^{K586E/M587E}$, or $GB2^{tripleE}$. Expression of single and double $GABA_{B2}$ mutants resulted in decreased functional responses compared with wt$GABA_{B2}$, with the $GABA_{B2}{}^{tripleE}$ mutant showing no response at all. *d*, Representative FLIPR analysis of intracellular Ca^{2+} changes after GABA stimulation of cells transiently transfected with G_{qi5}, wt$GABA_{B1}$ (*GB1*), and wt$GABA_{B2}$ (*GB2*), $GB2^{K586A}$, $GB2^{K590A}$, $GB2^{K586A/M587A}$, or $GB2^{tripleA}$. Single and double $GABA_{B2}$ mutants resulted in decreased functional responses compared with wt-$GABA_{B2}$, with the $GABA_{B2}{}^{tripleA}$ mutant completely unresponsive to GABA. *e*, Mutation of residues within the second intracellular loop of $GABA_{B2}$ do not affect agonist or antagonist ligand binding. Membrane homogenates prepared from cells transiently transfected with wt$GABA_{B1}$ (*GB1*) and wt$GABA_{B2}$ (*GB2*), $GB2^{K586E}$, $GB2^{K590E}$, $GB2^{K586E/M587E}$, or $GB2^{tripleE}$ specifically bound [^{3}H]CGP54626. This could be completely displaced by GABA (10 mM). Data are expressed as means ± SEM (n = 3).

Mutations in the second intracellular loop of $GABA_{B2}$ have no effect on agonist or antagonist binding

Having shown a marked effect on $GABA_B$ receptor signaling after mutation of selected residues within the second intracellular loop of $GABA_{B2}$, we wanted to see whether the agonist and antagonist binding properties of the mutant receptor heterodimer were consistent with that of the wild-type receptor. Analysis of membrane homogenates expressing each $GABA_{B2}$ mutant in combination with wt$GABA_{B1}$ had no effect on the ability of GABA to displace the antagonist CGP54626 in competition binding assays. The potency of GABA observed in homogenates prepared from cells expressing $GABA_{B2}{}^{K586E}$, $GABA_{B2}{}^{K590E}$, $GABA_{B2}{}^{K586E/M587E}$, or $GABA_{B2}{}^{tripleE}$ with wt$GABA_{B1}$ subunit was not significantly different from that observed when the wild-type $GABA_{B2}$ subunit was coexpressed with the $GABA_{B1}$ subunit (pK_i values of 4.57 ± 0.17, 4.22 ± 0.05, 4.48 ± 0.22, 4.84 ± 0.54, and 4.37 ± 0.12, respectively; n = 3) (Fig. 2*e*). B_{max} values of [^{3}H]CGP54626 specifically bound did not differ significantly between wild-type and mutants and ranged from 5.6 ± 3.3 to 8.3 ± 3.7 pmol/mg protein.

Negatively charged amino acids in the second intracellular loop of $GABA_{B1}$ are not critical for $GABA_B$ receptor signaling through G_{qi5}

The demonstration that mutation of specific residues within the second intracellular loop of $GABA_{B2}$ can have pronounced effects on $GABA_B$ signaling through G_{qi5} led us to examine the functional importance of similarly positioned residues within the $GABA_{B1}$ subunit. We therefore studied the consequences of mutating the stretch of negative amino acids found in the second intracellular loop of $GABA_{B1}$ and in a similar position to those residues mutated previously in $GABA_{B2}$ (Fig. 3*a*). Each of the mutants $GABA_{B1}{}^{E579K}$, $GABA_{B1}{}^{E583K}$, $GABA_{B1}{}^{E579K/E580K}$, and $GABA_{B1}{}^{tripleK}$ were transfected into HEK293 cells with and without wt$GABA_{B2}$. In the absence of $GABA_{B2}$, neither wt or mutant $GABA_{B1}$ variants were expressed at the cell surface (Fig. 3*b* and data not shown). However, when coexpressed with the $GABA_{B2}$ subunit, cell surface expression of all $GABA_{B1}$ mutants was observed as expected (Fig. 3*b* and data not shown). These experiments therefore suggest that mutation of these acidic residues in the second intracellular loop of $GABA_{B1}$ do not affect trafficking of the subunit to the surface by $GABA_{B2}$. We then tested the ability of these constructs to activate G_{qi5} in response to GABA. Coexpression of $GABA_{B2}$ with $GABA_{B1}{}^{E579K}$, $GABA_{B1}{}^{E579K/E580K}$, $GABA_{B1}{}^{E583K}$, or $GABA_{B1}{}^{tripleK}$ produced no significant changes in functional response when compared with coexpression with the wt$GABA_{B1}$ subunit (pEC_{50} values for wt$GABA_{B1}$, 7.19 ± 0.06, n = 36; $GABA_{B1}{}^{E579K}$,

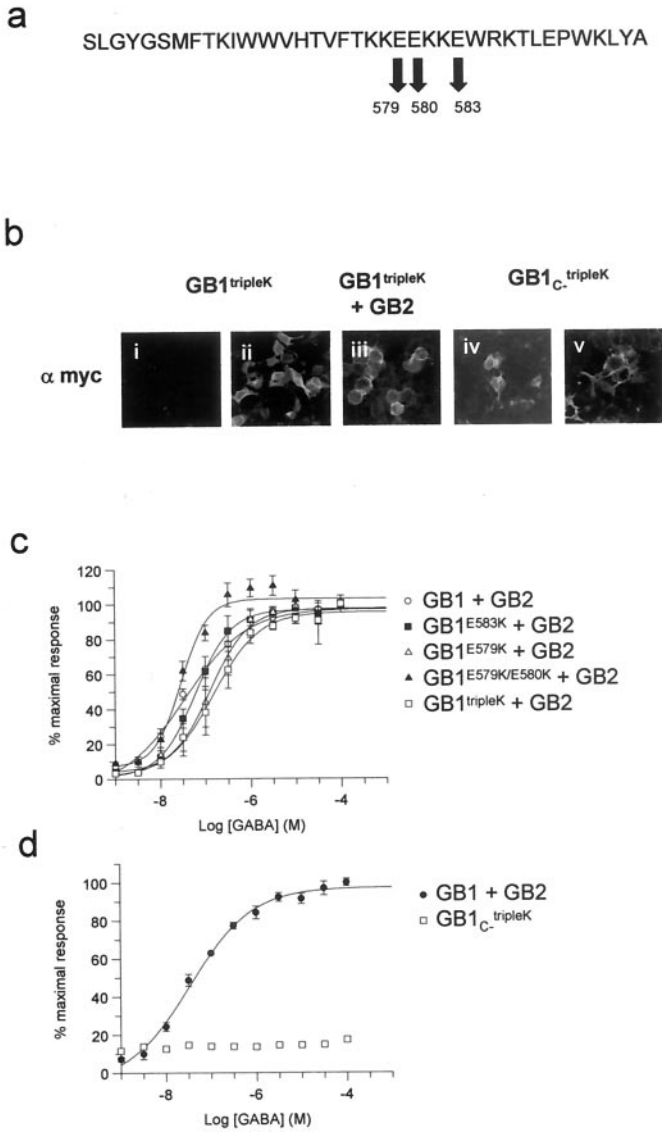

Figure 3. Acidic residues within the second intracellular loop of $GABA_{B1}$ are not critical for agonist-induced $GABA_B$ receptor signaling. *a*, Schematic representation of $GABA_{B1}$ il2. *Arrows* indicate residues mutated. *b*, HEK293 cells were transiently transfected with wt$GABA_{B1}$ (*GB1*), $GB1^{E579K}$, $GB1^{E583K}$, $GB1^{E579K/E580K}$, $GB1^{tripleK}$, or $GB1_{C\text{-}}{}^{tripleK}$ alone or with wt$GABA_{B2}$. Cell surface expression of $GABA_{B1}$ subunits was examined by immunofluorescence using an anti-myc antibody in the absence (*i*, *iii*, *iv*) or presence (*ii*, *v*) of a permeabilizing detergent. $GABA_{B1}$ is only found at the cell surface when expressed with $GABA_{B2}$, as shown with the $GABA_{B1}{}^{tripleK}$ construct (*i–iii*). The C-terminally truncated $GABA_{B1}$ is expressed at the cell surface in the absence of $GABA_{B2}$, as shown with the $GABA_{B1C\text{-}}{}^{tripleK}$ mutant (*iv*, *v*). *c*, Representative FLIPR analysis of cells transiently transfected with G_{qi5}, wt$GABA_{B2}$ (*GB2*), and wt$GABA_{B1}$ (*GB1*), $GB1^{E579K}$, $GB1^{E583K}$, $GB1^{E579K/E580K}$, or $GB1^{tripleK}$. No differences in functional GABA responses were observed between mutant and wild-type $GABA_{B1}$ subunits. *d*, Representative FLIPR analysis of cells transiently transfected with G_{qi5} and $GB1_{C\text{-}}{}^{tripleK}$. Expression of $GB1_{C\text{-}}{}^{tripleK}$ alone did not give a functional response.

7.03 ± 0.13, $n = 8$; $GABA_{B1}{}^{E579K/E580K}$, 7.18 ± 0.16, $n = 8$; $GABA_{B1}{}^{E583K}$, 7.02 ± 0.13, $n = 7$; and $GABA_{B1}{}^{tripleK}$, 7.18 ± 0.16, $n = 7$) (Fig. 3*c*). This suggests that these acidic residues are not critical in the signaling of the $GABA_{B1}$ receptor dimer through G_{qi5}.

Addition of positively charged residues to the second intracellular loop of a cell surface-expressed $GABA_{B1}$ is not sufficient for receptor function in the absence of $GABA_{B2}$

Although mutation of acidic residues in the second intracellular loop of $GABA_{B1}$ appeared to have no functional effect with respect to heterodimer signaling, we also wanted to test the function of $GABA_{B1}$ mutants when expressed on the cell surface in the absence of $GABA_{B2}$. We and others have shown previously that removal of the C-terminal domain of the $GABA_{B1}$ subunit results in its cell surface expression in the absence of $GABA_{B2}$. However, despite this cell surface expression, the $GABA_{B1}$ subunit remains ineffective in coupling to downstream effector systems in the absence of $GABA_{B2}$ (Calver et al., 2000; Margeta-Mitrovic et al., 2000). In this set of experiments, we therefore produced four C-terminal truncates: $GABA_{B1C\text{-}}{}^{E579K}$, $GABA_{B1C\text{-}}{}^{E579K/E580K}$, $GABA_{B1C\text{-}}{}^{E583K}$, or $GABA_{B1C\text{-}}{}^{tripleK}$. Expression of these mutants showed that they were all able to reach the cell surface in the absence of $GABA_{B2}$ (Fig. 3*b* and data not shown). We then compared the ability of each $GABA_{B1}$ C-terminal truncate mutant to couple to G_{qi5} after stimulation by GABA. All of the $GABA_{B1}$ mutants tested were nonfunctional when expressed alone ($n = 4$) (Fig. 3*d* and data not shown). In contrast, expression of each truncated $GABA_{B1}$ mutant gave a robust signal when coexpressed with $GABA_{B2}$, no different to that seen with wt$GABA_{B1}$ (data not shown). This suggests that removal of negatively charged glutamic acid residues within the second intracellular loop of $GABA_{B1}$ is not sufficient to allow signaling and strengthens the argument that the $GABA_{B2}$ subunit is absolutely necessary for G-protein signaling.

Positively charged amino acids in the second intracellular loop of $GABA_{B1}$ are not critical for $GABA_B$ receptor signaling through G_{qi5}

We also noted when aligning the $GABA_{B1}$ and $GABA_{B2}$ il2 sequences that there were five positively charged lysine residues in this region of $GABA_{B1}$, although they do not directly align with the positive residues within il2 of $GABA_{B2}$ that we implicated in G-protein coupling. We therefore wanted to exclude the possibility that these amino acids might be involved in receptor signaling. We studied the effects of mutating the five lysine residues in il2 of $GABA_{B1}$ to neutral alanines and/or negatively charged glutamates, both in pairs and all five together (Fig. 4*a*). Each of the mutants $GABA_{B1}{}^{K577/578A}$, $GABA_{B1}{}^{K581/582A}$, $GABA_{B1}{}^{KQA}$, and $GABA_{B1}{}^{KQE}$ were transfected into HEK293 cells together with $GABA_{B2}$. This resulted in cell surface expression of all of the $GABA_{B1}$ mutants as expected (Fig. 4*b*). We then tested the ability of these constructs to activate G_{qi5} in response to GABA. Coexpression of $GABA_{B2}$ with $GABA_{B1}{}^{K577/578A}$, $GABA_{B1}{}^{K581/582A}$, $GABA_{B1}{}^{KQA}$, or $GABA_{B1}{}^{KQE}$ resulted in the expression of functional $GABA_B$ receptors. Neither of the double mutations ($GABA_{B1}{}^{K577/578A}$ or $GABA_{B1}{}^{K581/582A}$) produced any significant changes in the potency of GABA when compared with the wild-type receptor, and although the potency of GABA at the quintuple mutant subunits ($GABA_{B1}{}^{KQA}$ and $GABA_{B1}{}^{KQE}$) was slightly lower than the wild type, this was a very minor effect when compared with the mutations in $GABA_{B2}$ (pEC_{50} values for wt$GABA_{B1}$, 7.19 ± 0.06, $n = 36$; $GABA_{B1}{}^{K577/578A}$, 7.28 ± 0.04, $n = 9$; $GABA_{B1}{}^{K581/582A}$, 7.17 ± 0.08, $n = 9$; $GABA_{B1}{}^{KQA}$, 6.74 ± 0.08, $n = 12$; and $GABA_{B1}{}^{KQE}$, 6.76 ± 0.03, $n = 9$) (Fig. 4*c*). These data suggest that the basic residues

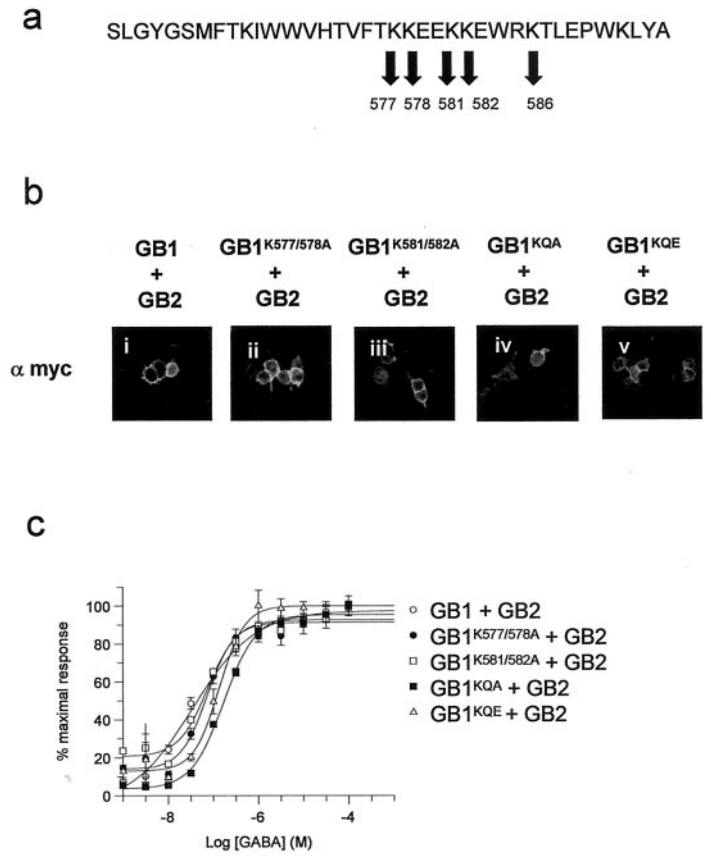

Figure 4. Basic residues within the second intracellular loop of $GABA_{B1}$ are not critical for agonist-induced $GABA_B$ receptor signaling. *a*, Schematic representation of $GABA_{B1}$ il2. *Arrows* indicate residues mutated. *b*, HEK293 cells were transiently transfected with wt$GABA_{B2}$ (*GB2*) and wt$GABA_{B1}$ (*GB1*), $GB1^{K577/578A}$, $GB1^{K581/582A}$, $GB1^{KQA}$, or $GB1^{KQE}$. $GABA_{B1}$ is only found at the cell surface when expressed with $GABA_{B2}$, as shown by immunofluorescence using an anti-myc antibody in the absence (*i–v*) of a permeabilizing detergent. *c*, Representative FLIPR analysis of cells transiently transfected with G_{qi5}, wt$GABA_{B2}$ (*GB2*), and wt$GABA_{B1}$ (*GB1*), $GB1^{K577/578A}$, $GB1^{K581/582A}$, $GB1^{KQA}$, or $GB1^{KQE}$. Both mutant and wild-type $GABA_{B1}$ subunits are functional when coexpressed with wt$GABA_{B2}$.

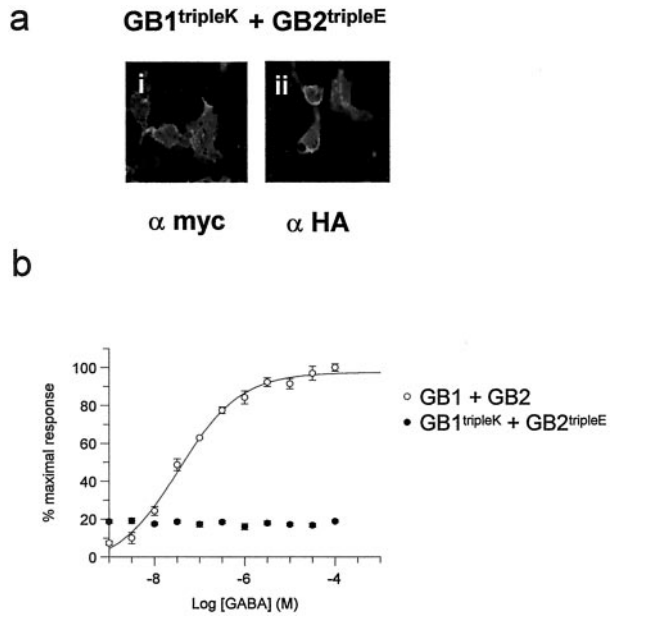

Figure 5. Loss of functional coupling after exchange of $GABA_{B1}$ and $GABA_{B2}$ second intracellular loop charged residues. *a*, HEK293 cells were transiently transfected with $GABA_{B1}{}^{tripleK}$ (*GB1tripleK*) and $GABA_{B2}{}^{tripleE}$ (*GB2tripleE*). Cell surface expression of $GABA_B$ subunits was examined by immunofluorescence using an anti-myc antibody for $GB1^{tripleK}$ (*i*) and an anti-HA antibody for $GB2^{tripleE}$ (*ii*). *b*, Representative FLIPR analysis showing no functional response to GABA in cells transiently transfected with G_{qi5}, $GB1^{tripleK}$, and $GB2^{tripleE}$.

in il2 of $GABA_{B1}$ are also not critical for the signaling of the $GABA_B$ receptor dimer through G_{qi5}.

Exchanging charged residues between the second intracellular loops of $GABA_{B1}$ and $GABA_{B2}$ abolishes $GABA_B$ receptor functional coupling to G-proteins

Because $GABA_{B2}{}^{tripleE}$ when coexpressed with the wild-type $GABA_{B1}$ produced a nonfunctional receptor, we coexpressed $GABA_{B2}{}^{tripleE}$ with $GABA_{B1}{}^{tripleK}$ in HEK293 cells. Despite both subunits being expressed at the cell surface (Fig. 5*a*), no functional response was detected in the Ca^{2+} mobilization assay ($n = 8$) (Fig. 5*b*). This suggests that $GABA_{B2}$ is essential for G-protein coupling in the $GABA_B$ receptor.

Amino acids in the second intracellular loop of $GABA_{B2}$ are critical for $GABA_B$ receptor signaling through endogenous G-proteins in superior cervical ganglion cells

It was important to determine that the functional effects observed using our $GABA_{B2}$ mutant constructs in conjunction with G_{qi5} could be reproduced in an effector system coupled to endogenously expressed G-proteins. We demonstrated previously that SCG neurons express only the $GABA_{B1}$ subunit. However microinjection of $GABA_{B2}$ constructs into these neurons results in the expression of functional $GABA_B$ receptors that inhibit Ca^{2+} channels (Filippov et al., 2000). Microinjecton of $GABA_{B2}{}^{K590E}$ and $GABA_{B2}{}^{tripleE}$ resulted in cell surface expression of $GABA_{B2}$ (Fig. 6*a*) and $GABA_{B1}$ subunits (data not shown). Expression of the wt$GABA_{B2}$ subunit and stimulation by baclofen resulted in an $\sim 53 \pm 2.02\%$ inhibition of voltage-activated Ca^{2+} currents (Fig. 6*b*,*c*), as measured 10 msec after the voltage pulse. Expression of $GABA_{B2}{}^{K590E}$ resulted in significantly reduced inhibition of Ca^{2+} currents when compared with wt-$GABA_{B2}$ ($42 \pm 1.92\%$) inhibition (Fig. 6*b–d*). In contrast to the other mutants studied, injection of the $GABA_{B2}{}^{tripleE}$ mutant resulted in no baclofen-stimulated inhibition of Ca^{2+} channel opening above that observed in mock-injected neurons. This demonstrates that the $GABA_{B2}{}^{tripleE}$ mutant, in conjunction with endogenous $GABA_{B1}$, forms a completely nonfunctional $GABA_B$ receptor (Fig. 6*b*,*c*).

The $GABA_{B2}$ subunit alone is unable to functionally respond to GABA

It is known that the $GABA_{B1}$ subunit contains a binding site for GABA in its N-terminal domain (Malitschek et al., 1999), but it is still unclear from published work as to whether $GABA_{B2}$ is capable of binding and responding to GABA on its own. We therefore generated a $GABA_{B2}$ stable cell line in HEK293 cells, in which we confirmed $GABA_{B2}$ expression on the cell surface by both immunocytochemistry and Western blotting (data not shown). We were unable to observe any [^{35}S]GTPγS binding during stimulation with GABA up to a concentration of 1 mM (Fig. 7*a*). Similarly, we were unable to demonstrate any inhibition

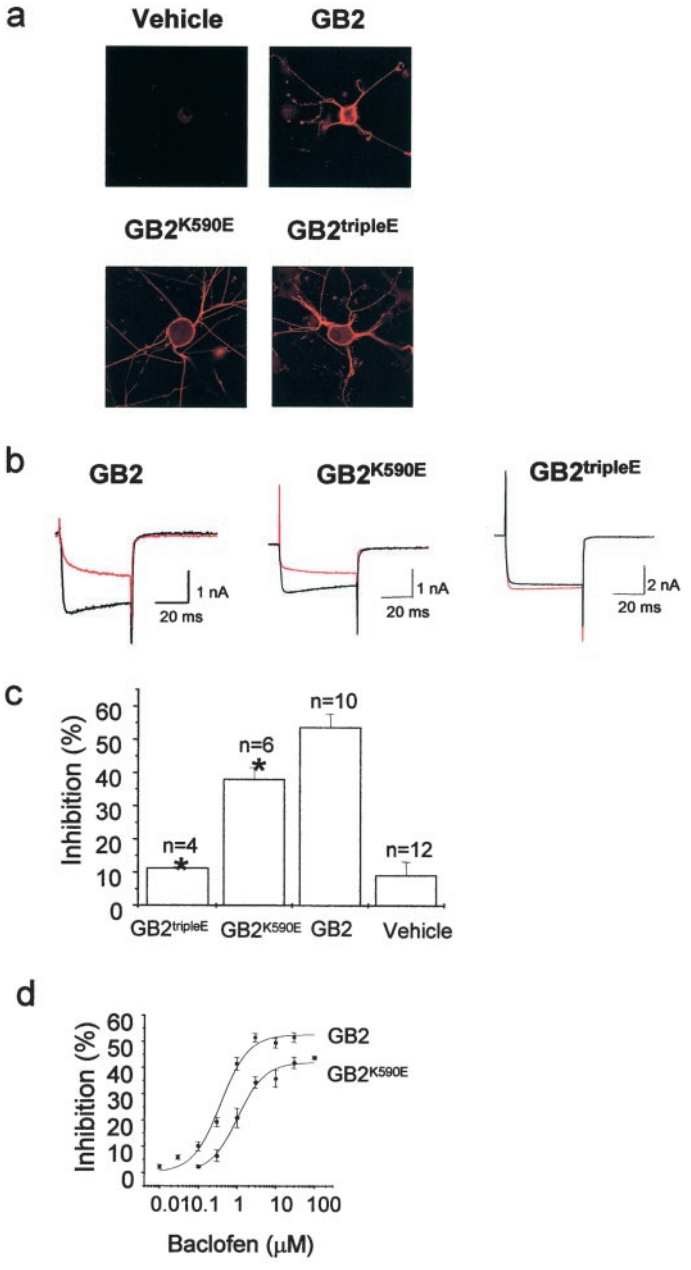

Figure 6. Coupling of $wtGABA_{B2}$ and $GABA_{B2}$ mutants to N-type Ca^{2+} channels in sympathetic neurons. *a*, Cell surface expression of wild-type and mutant $GABA_{B2}$ subunits examined by immunofluorescence using an anti-HA antibody. *b*, Currents were recorded by stepping for 100 msec every 20 sec from −90 to 0 mV and leak-corrected by subtracting currents remaining after substituting 5 mM Co^{2+} for Ba^{2+}. Records show superimposed leak-subtracted currents in the absence (*black line*) and presence (*red line*) of 50 μM baclofen for neurons injected 16–24 hr before recordings with 100–150 ng/μl $wtGABA_{B2}$ (*GB2*), $GB2^{K590E}$, or $GB2^{tripleE}$. *c*, Bar charts show the mean inhibition of I_{Ba} amplitude by 50 μM baclofen, 10 msec after voltage stepping, in neurons injected with GB2, $GB2^{K590E}$, or $GB2^{tripleE}$. Error bars show SEM; *n* indicates number of cells. Note that $GABA_{B2}{}^{K590E}$ still mediates inhibition of Ca^{2+} channel current, whereas $GABA_{B2}{}^{tripleE}$ does not couple to Ca^{2+} channels. *d*, Plots show concentration dependence of Ca^{2+} current inhibition by baclofen in SCG neurons injected with GB2 ($n = 5$) or $GB2^{K590E}$ ($n = 3$). Curves were fitted to pooled data points (mean ± SEM) using Origin 5 software to the Hill equation $y = y_{max\ [infl]} \cdot x^{nH} / (x^{nH} + K^{nH})$, where *y* is observed percentage of inhibition, y_{max} is the extrapolated maximal percentage of inhibition, *x* is baclofen concentration (micromolar), *K* is IC_{50} (micromolar), and nH is the Hill coefficient. For $GABA_{B2}{}^{K590E}$, IC_{50} of 1.04 ± 0.19 μM; nH of 1.23 ± 0.24; percentage of maximal inhibition, 42.03 ± 1.92%. For $GABA_{B2}$, IC50 of 0.38 ± 0.06 μM; nH of 1.21 ± 0.19; percentage of maximal inhibition, 52.55 ± 2.02%. Note that $GABA_{B2}{}^{K590E}$ inhibits Ca^{2+} current less effectively than $GABA_{B2}$ and that plots could not be made for $GABA_{B2}{}^{tripleE}$ because there was no detectable functional response.

of forskolin-stimulated adenylate cyclase activity in these cells in response to GABA at a concentration of up to 100 μM (Fig. 7*b*). In cell lines expressing both $GABA_{B1}$ and $GABA_{B2}$ on the other hand, we were readily able to demonstrate functional coupling using both of these techniques with the pharmacology expected from recombinant $GABA_B$ receptors (Fig. 7*a*,*b*).

The N-terminal domain of $GABA_{B2}$ is also necessary for normal $GABA_B$ receptor signaling

To investigate whether the N-terminal domain of $GABA_{B2}$ is important in normal $GABA_B$ receptor function, we made a chimeric $GABA_{B2}$ construct by replacing the N-terminal binding domain of $GABA_{B2}$ with that of $GABA_{B1}$. Expression of this $GABA_{B1N/2}$ subunit was clearly observed on the cell surface, and furthermore it retained the ability to traffic $wtGABA_{B1}$ to the cell surface (Fig. 7*c*). However, functional analysis of cells expressing both $GABA_{B1N/2}$ and $wtGABA_{B1}$ exhibited a complete lack of response to GABA ($n = 4$) (Fig. 7*d*). This suggests that the N-terminal domain of $GABA_{B2}$ may also be important in the mediation of receptor responses to GABA, despite the fact that this subunit does not actually bind GABA.

DISCUSSION

$GABA_B$ receptors are the only members of the type C family of GPCRs that have been shown to function as heterodimers. They are also distinct from other reported GPCR heterodimers in which both members of the heterodimer complex are able to form functional receptors when expressed as monomers (Jordan and Devi, 1999; AbdAlla et al., 2000; Gines et al., 2000; Gomes et al., 2000; Rocheville et al., 2000). In this study, we wanted to further analyze the role of each $GABA_B$ subunit with respect to G-protein coupling and signal transduction, focusing on residues within the second and third intracellular loops. Residues within these domains have been demonstrated previously to be critical for G-protein interactions in a number of receptors. More specifically, recent studies on the functional roles of the cytoplasmic domains of rhodopsin have suggested that the third intracellular loop contains sites important in determining G-protein specificity, whereas the second intracellular loop contains regions essential for G-protein activation (Yamashita et al., 2000). Sequence comparison of residues in il2 and il3 for both the $GABA_B$ subunits and the related mGluRs highlighted clear differences between $GABA_{B1}$ and $GABA_{B2}$ of potential relevance for G-protein coupling (Fig. 1*a*), suggesting that it may be $GABA_{B2}$ rather than $GABA_{B1}$ that is involved in the coupling of the $GABA_B$ receptor to G-proteins and downstream signaling. Furthermore, comparison of 46 il2 sequences from various species demonstrated that all of the mGluRs and $GABA_{B2}$ contained fewer than two negatively charged residues within this loop. This is in sharp contrast to $GABA_{B1}$, which contains four negatively charged residues. This may be of particular relevance in the context of modeling studies by Fannelli et al. (1998), who investigated the electrostatic complimentarity of receptor-G-protein complexes. These studies favor a model whereby "opening" of the cytosolic domains of the receptor allows for an interaction between the electrostatically positive surface of the domains of the

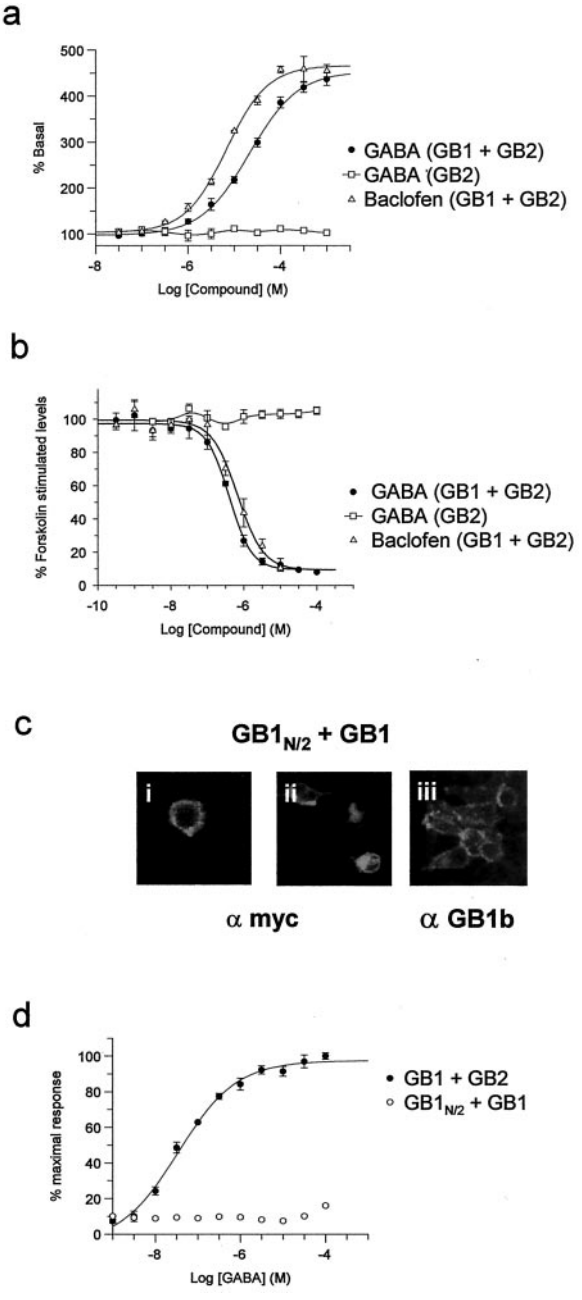

Figure 7. $GABA_{B2}$ expressed alone is unable to functionally couple to adenylate cyclase or bind GTPγS in response to GABA, but substitution of the $GABA_{B2}$ N terminus with that of $GABA_{B1}$ abolishes $GABA_B$ receptor function. *a*, Membranes prepared from cells expressing either $GABA_{B2}$ (*GB2*) alone (*squares*) or both $GABA_{B1}$ (*GB1*) and $GABA_{B2}$ together (*circles*, *triangles*) were incubated with either GABA (*circles*, *squares*) or baclofen (*triangles*) and subsequently with [^{35}S]GTPγS. Data are presented as the percentage of increase in [^{35}S]GTPγS binding over basal in response to agonist and are expressed as means ± SEM ($n = 3$). *b*, Cells expressing either $GABA_{B2}$ alone or $GABA_{B1}$ and $GABA_{B2}$ together (*symbols* and legend as above) were incubated with forskolin and then either GABA or baclofen. Data are presented as the percentage of forskolin-stimulated cAMP levels remaining after incubation with agonist and are expressed as means ± SEM ($n = 3$). *c*, HEK293 cells were transiently transfected with $GABA_{B1N/2}$ ($GB1_{N/2}$) and wt$GABA_{B1}$ (*GB1*), and cell surface expression of $GABA_B$ subunits was examined by immunofluorescence using either an anti-myc antibody for $GABA_{B1N/2}$ in the absence (*i*) or presence (*ii*) of detergent or an anti-$GABA_{B1}$ antibody for wt$GABA_{B1}$ (*iii*). *d*, Representative FLIPR analysis showing no functional response to GABA in cells transiently transfected with G_{qi5}, $GABA_{B1N/2}$ ($GB1_{N/2}$), and wt$GABA_{B1}$ (*GB1*).

receptor and the negatively charged surface of the G_α subunit (Higgs and Reynolds, 2001). In this respect, it is of interest that the electrostatic potential of G_o, reported to be the predominant G-protein interacting with $GABA_B$ receptors (Leaney and Tinker, 2000), is proposed to be one of the most negative of the G_α subunits (C. Reynolds, personal communication). Based on these observations, we can speculate that the negatively charged residues of the $GABA_{B1}$ subunit would make it a less attractive candidate for G-protein coupling than $GABA_{B2}$.

To determine the functional role that il2 residues play in $GABA_B$ receptor function, we made a series of amino acid substitution mutants at K^{586}, M^{587}, and K^{590} in $GABA_{B2}$. Single or double substitutions of these residues to glutamate or alanine did not affect the ability of $GABA_{B2}$ to reach the cell surface itself or to traffic $GABA_{B1}$ to the cell surface but led to a significant reduction in agonist potency when compared with the wild-type receptor heterodimer. In addition, when all three residues were substituted by glutamates or alanines, it resulted in a receptor that was completely unresponsive to agonists. These changes in agonist efficacy were not attributable to aberrant folding of the receptor subunits or changes in binding affinity because both agonist and antagonist binding was unaltered when compared with wild-type receptor. Moreover, our binding data were consistent with immunocytochemical data and suggested that there was no significant effect on receptor expression levels. Importantly, these mutants also interfered with the physiological coupling of $GABA_B$ receptors to Ca^{2+} channels in SCG neurons, confirming that mutation of these residues in il2 of $GABA_{B2}$ was sufficient to abolish $GABA_B$ receptor signaling. These data therefore identify key residues important in G-protein coupling and activation of the receptor heterodimer and is in agreement with previous proposals suggesting that the heptahelical domains of $GABA_{B2}$, and not $GABA_{B1}$, contain these molecular determinants (Calver et al., 2001; Galvez et al., 2001).

To further investigate the role of il2 residues in the heterodimer, we substituted the corresponding negatively charged residues in $GABA_{B1}$, E^{579}, E^{580}, and E^{583}, to lysines. These $GABA_{B1}$ mutants were all expressed on the cell surface after coexpression with $GABA_{B2}$, and all responded to agonist as effectively as the wild-type receptor heterodimer. Similarly, $GABA_{B1}$ mutants in which all five positively charged lysine residues in this region were changed to either neutral or negatively charged amino acids are completely functional within heterodimers with wild-type $GABA_{B2}$. Furthermore, a number of other $GABA_{B1}$ mutants we expressed all showed a complete lack of responsiveness to GABA in the absence of $GABA_{B2}$. Although this data does not completely discount the possibility that G-proteins may be interacting with il2 of the $GABA_{B1}$ subunit, it does suggest that it is less likely that there are electrostatic interactions between $GABA_{B1}$ and G-proteins. A recent study however has reported that replacement of the entire seven transmembrane and intracellular domains of $GABA_{B1}$ with that of $GABA_{B2}$ (termed GB1/2) reduces the efficacy of G-protein coupling to a "GB1/2 GB2" receptor (Galvez et al., 2001). Although this may be indicative of a modulatory role for $GABA_{B1}$ in G-protein coupling, we would suggest that it is more likely as a result of less efficient signaling between $GABA_{B1}$ and its "downstream" partner, $GABA_{B2}$.

Molecular modeling studies have identified residues in the $GABA_{B1}$ subunit that are critical for binding of $GABA_B$ agonists and antagonists, and these residues are absent from the $GABA_{B2}$ subunit (Galvez et al., 2000a,b). In this study, we demonstrated

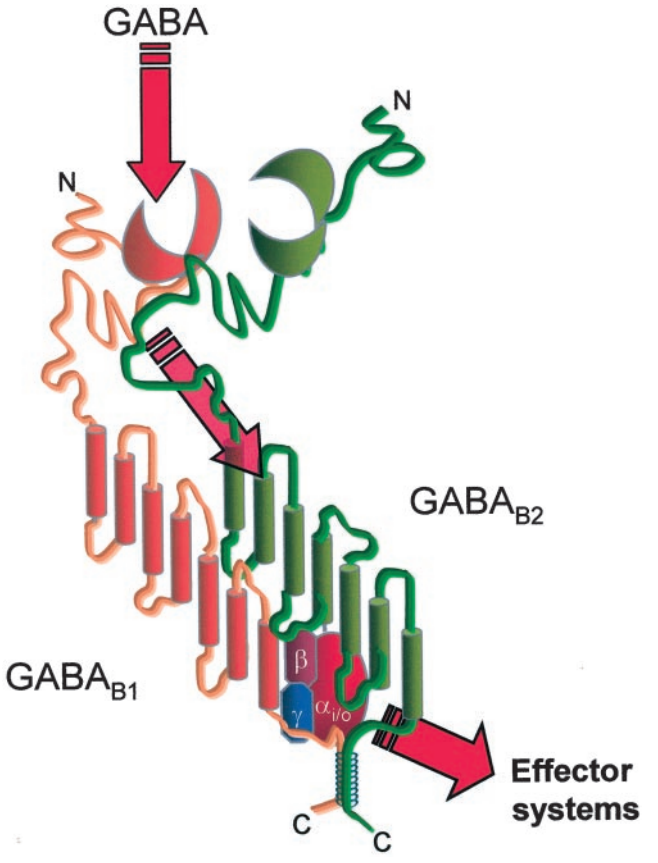

Figure 8. Proposed sideways signaling mechanism for the $GABA_B$ receptor heterodimer. $GABA_{B1}$ (*orange*) and $GABA_{B2}$ (*green*) subunits form heterodimers via C-terminal coiled-coil interactions, as well as N-terminal and possibly transmembrane domain interactions. The agonist GABA binds to the ligand binding domain within the $GABA_{B1}$ N terminus, resulting in a conformational change in the $GABA_{B2}$ subunit. This activated state of the receptor heterodimer allows recruitment of G-proteins, at least in part via an interaction with the positively charged residues in il2 of $GABA_{B2}$ and subsequent activation of downstream signal transduction cascades.

that $GABA_{B2}$ expressed in the absence of $GABA_{B1}$ in HEK293 cells is unable to respond functionally to GABA, by either inhibition of adenylate cyclase activity or binding of [^{35}S]GTPγS. However, the absence of a ligand binding site does not necessarily mean that $GABA_{B2}$ is not important in signal transduction. Indeed, the recent solving of the crystal structures for the N-terminal binding domain of mGluR1 demonstrate that movements between all four globular lobes of the N-terminal domains of the dimer are likely to cause shifts in transmembrane and intracellular domains, resulting in receptor activation (Kunishima et al., 2000). These findings may help to explain results from this and other studies in which deletion or substitution of substantial parts of the $GABA_{B2}$ N-terminal domain results in a complete loss of $GABA_B$ receptor function (Jones et al., 2000; Galvez et al., 2001). This therefore suggests that the N-terminal domain of the $GABA_{B2}$ subunit, although unable to bind GABA itself, may instead be involved in the conformational changes induced after ligand binding to the $GABA_{B1}$ subunit.

In summary, our study has determined three residues within il2 of the $GABA_{B2}$ subunit critical for G-protein signaling of the $GABA_B$ receptor heterodimer, demonstrating that this signaling absolutely requires the presence of the $GABA_{B2}$ protein. Although our data do not rule out a possible involvement of $GABA_{B1}$ in the signaling process, these studies, together with current knowledge of this receptor heterodimer, strongly suggest a novel mechanism of "sideways" signal transduction, whereby agonist binds to the $GABA_{B1}$ subunit, resulting in a conformational change that is in some way passed on to the $GABA_{B2}$ subunit, perhaps through its N-terminal and/or transmembrane domains. This in turn results in the receptor forming an activated state suitable for G-protein recruitment, via a direct interaction with $GABA_{B2}$, and thus downstream signal transduction (Fig. 8). Such novel sideways signaling may allow increased complexity of receptor signal transduction, which may also be applicable to other GPCR heterodimers.

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