Heteromeric Assembly of GABA$_B$R1 and GABA$_B$R2 Receptor Subunits Inhibits Ca^{2+} Current in Sympathetic Neurons

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Neuronal GABA$_B$ receptors regulate calcium and potassium currents via G-protein-coupled mechanisms and play a critical role in long-term inhibition of synaptic transmission in the CNS. Recent studies have demonstrated that assembly of GABA$_B$ receptor GABA$_B$R1 and GABA$_B$R2 subunits into functional heterodimers is required for coupling to potassium channels in heterologous systems. However whether heterodimerization is required for the coupling of GABA$_B$ receptors to effector systems in neurons remains to be established. To address this issue, we have studied the coupling of recombinant GABA$_B$ receptors to endogenous Ca^{2+} channels in superior cervical ganglion (SCG) neurons using nuclear microinjection to introduce both sense and antisense expression constructs. Patch-clamp recording from neurons injected with both GABA$_B$R1a/1b and GABA$_B$R2 cDNAs or with GABA$_B$R2 alone produced marked baclofen-mediated inhibition of Ca^{2+} channel currents via a *per-tussis* toxin-sensitive mechanism. The actions of baclofen were blocked by CGP62349, a specific GABA$_B$ antagonist, and were voltage dependent. Interestingly, SCGs were found to express abundantly GABA$_B$R1 but not GABA$_B$R2 at the protein level. To determine whether heterodimerization of GABA$_B$R1 and GABA$_B$R2 subunits was required for Ca^{2+} inhibition, the GABA$_B$R2 expression construct was microinjected with a GABA$_B$R1 antisense construct. This resulted in a dramatic decrease in the levels of the endogenous GABA$_B$R1 protein and a marked reduction in the inhibitory effects of baclofen on Ca^{2+} currents. Therefore our results suggest that in neurons heteromeric assemblies of GABA$_B$R1 and GABA$_B$R2 are essential to mediate GABAergic inhibition of Ca^{2+} channel currents.

Key words: GABA$_B$R1; GABA$_B$R2; Ca^{2+} channel; sympathetic neuron; antisense; G-protein

GABA$_B$ receptors play a critical role in long-term inhibition of synaptic transmission in the CNS (Bowery, 1993; Mott and Lewis, 1994). Inhibition is mainly achieved via modulation of neurotransmitter release from presynaptic terminals and hyperpolarization of postsynaptic membranes. Although activation of inwardly rectifier potassium channels (GIRKs) is thought to be responsible for a GABA$_B$-mediated membrane hyperpolarization, inhibition of presynaptic N-type Ca^{2+} channels is thought to be responsible for modulation of neurotransmitter release (Mott and Lewis, 1994; Kaupmann et al., 1998; Takahashi et al., 1998). The regulation of GABA$_B$ receptor function has been implicated in cognition enhancement and induction of long-term potentiation (Davies et al., 1991; Olpe et al., 1993). In addition, involvement of the GABA$_B$ receptor in a number of diseases of the CNS such as epilepsy, anxiety, depression, and cognitive deficits make it an attractive target for therapeutic agents (Bittiger et al., 1993; Kerr and Ong, 1995; Dichter, 1997).

The first GABA$_B$ receptors, GABA$_B$R1a and GABA$_B$R1b, were cloned from a mouse cortical and cerebellar cDNA library (Kaupmann et al., 1997). They exhibited a low affinity for agonists and did not couple efficiently to neuronal Ca^{2+} channels when expressed heterologously in sympathetic neurons (Couve et al., 1998). Subcellular distribution of epitope-tagged GABA$_B$R1 demonstrated that recombinant receptors failed to reach the cell surface and were retained in intracellular compartments when expressed in a variety of neuronal and non-neuronal cell types. These results provided an explanation for the lack of function of recombinant GABA$_B$R1 receptors in coupling to other effector systems such as K$^+$ channels (Couve et al., 1998). Recently GABA$_B$R2, a second receptor ~35% identical to GABA$_B$R1, has been identified by several laboratories (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). Unexpectedly for a G-protein-coupled receptor, heteromeric assembly of GABA$_B$R1 and GABA$_B$R2 has been shown to be necessary for coupling to adenylyl cyclase and activation of GIRKs in *Xenopus* oocytes and human embryonic kidney 293 (HEK293) cells (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). However, it has yet to be determined whether heterodimer formation is required for coupling to their effector systems (such as Ca^{2+} channels) in neurons.

Here we demonstrate that the heteromeric assembly of GABA$_B$R1 and GABA$_B$R2 receptor subunits is required to allow GABA$_B$ receptor-induced inhibition of Ca^{2+} currents in rat superior cervical ganglion (SCG) neurons.

MATERIALS AND METHODS

Neuron preparation and cDNA injection. Neuron isolation and injection procedures have been described previously in detail (Filippov et al., 1997, 1998; Couve et al., 1998). Briefly, single 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 of the GABA$_B$ receptor subunits. Plasmids were stored at 4°C as 1 μg/μl stock solutions in 10 mM TRIS and 1 mM EDTA, pH 8, and injected at a final pipette concentration of 0.15–0.5 μg/μl. (Experimental data were indistinguishable over this range of concentrations for GABA$_B$R1a and GABA$_B$R1b.) Neurons were co-injected with cDNA for the "enhanced" S65T mutant of jellyfish green fluorescent protein (GFP) at concentrations of 0.01–0.5 μg/μl for later identification of the cells with successfully expressed cDNAs. For controls, neurons were injected with GFP cDNA alone or not injected at all. After injections, cells were incubated for 14–48 hr in a humidified incubator (5% CO$_2$/95% O$_2$) at 37°C. Injected neurons with successfully expressed cDNAs were identified as bright fluorescent cells using an inverted microscope (Diaphot 200; Nikon) equipped with an epifluorescent block N B2E (Nikon). Electrophysiological recordings were routinely made 16–20 hr after injection at room temperature (20°C). In some experiments, recordings were made 40 hr after injections. Where indicated, neurons were incubated for 12–16 hr with _pertussis_ toxin (PTX; 0.5 μg/μl) added to the culture media.

Plasmids. A cytomegalovirus (CMV) promoter-based mammalian expression vector containing a myc-tagged version of the rat GABA$_B$R1a receptor has been described previously (Couve et al., 1998). The GABA$_B$R1a antisense cDNA was obtained by subcloning the sequence of the complete receptor in the opposite orientation with its 3′ end facing the CMV promoter. A GABA$_B$R2 receptor plasmid was obtained by subcloning an ~3.5 kb _Xba_I containing the sequence of the rat GABA$_B$R2 receptor from pC1-Neo/GABA$_B$R2 (kindly provided by Dr. Bernhard Bettler) into a CMV promoter-based mammalian expression vector.

Antibodies. A guinea pig pan antibody directed against the C-terminal domain of GABA$_B$R1a was obtained from Chemicon International. A rabbit antibody specific for GABA$_B$R1a was raised against the peptide CHPPWEGGIRYRGLTRDQVK from the N-terminal domain of GABA$_B$R1a conjugated with keyhole limpet hemocyanin and was immunopurified using the same peptide. A rabbit antibody directed against the C-terminal domain of GABA$_B$R2 was kindly provided by Dr. Bernhard Bettler. MAP2 monoclonal antibody was obtained from Sigma (St. Louis, MO). Monoclonal protein disulfide isomerase (DPI) antibody was purchased from Stress Gen. Synaptophysin antibody was kindly provided by Dr. Dan Cutler. Texas Red- and fluorescein-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

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). Cells were superfused (20–25 ml/sec) with a solution consisting of 120 mM tetraethylammonium chloride, 3 mM KCl, 1.5 mM MgCl$_2$, 5 mM BaCl$_2$, 10 mM HEPES, 11.1 mM glucose, and 0.5 μM tetrodotoxin. The pH was adjusted to 7.35 with NaOH. Patch electrodes (2–3 MΩ) were filled with a solution containing 110 mM CsCl, 3 mM MgCl$_2$, 40 mM HEPES, 3 mM EGTA, 2 mM Na$_2$ATP, and 0.5 mM Na$_2$GTP, with pH adjusted to 7.4 with CsOH. Neurons were voltage-clamped using a discontinuous ("switching") amplifier (Axoclamp 2B) sampling voltage at 6–8 kHz (50% duty cycle). Commands were generated via a Digidata 1200 interface using "Clamp 6" computer software (Axon Instruments, Foster City, CA). Ca^{2+} channel currents were routinely evoked every 20 sec with a 100 msec depolarizing rectangular test pulse to 0 mV from a holding potential of −90 mV. Currents were digitized and stored on a computer for later analysis using Clamp 6 software. Ca^{2+} channel current amplitudes were measured isochronally 10 msec from the onset of the rectangular test pulse, i.e., near the peak of the control current. To eliminate leak currents, Co^{2+} was substituted for Ba^{2+} in the external solution at the end of each experiment, blocking all Ca^{2+} channel currents, and the residual current was digitally subtracted from the corresponding currents in the Ba^{2+} solution. As reported previously (Filippov et al., 1997, 1998), currents were primarily N-type with negligible contribution by L-type channels.

Data were presented as means ± SEM as appropriate. Student's test (unpaired) was applied to determine statistical significance. The difference was considered significant if $p \leq 0.05$.

Baclofen and norepinephrine were purchased from Sigma. PTX was obtained from Porton Products (Dorset, UK).

Protein extracts, immunoblots, and immunofluorescence. Immunofluorescence and immunoblots were performed as described previously (Couve et al., 1998). For preparation of brain membranes, fresh rat brains were homogenized in 10 vol of 5 mM Tris-Cl, pH 7.4, 0.32 M sucrose, and a cocktail of protease inhibitors with 50 strokes using a glass and Teflon homogenizer. The suspension was centrifuged at 1000 $\times$ _g_ for 15 min. The pellet was discarded, and the resulting supernatant was centrifuged at 17000 $\times$ _g_ for 30 min. The pellet was washed twice in 50 mM Tris-Cl, pH 7.4, and resuspended at ~5 mg/ml in the same buffer. Aliquots were frozen and stored at −20°C until used. Extracts from superior cervical ganglia were prepared by homogenizing ganglia in RIPA buffer containing 50 mM Tris-Cl, 1 mM EDTA, 2 mM EGTA, 10 mM Na$^+$ pyrophosphate, 1 mM Na$^+$ orthovanadate, 50 mM NaF, 150 mM NaCl, 1% NP-40, 0.5% deoxychoic acid 0.1% SDS, and a cocktail of protease inhibitors. All extracts were stored at −20°C and mixed with SDS sample buffer before loading onto SDS-PAGE.

RESULTS

Heterologously expressed GABA$_B$R1 and GABA$_B$R2 or GABA$_B$R2 but not GABA$_B$R1 couple to N-type Ca^{2+} channels in sympathetic neurons

It has been reported previously that baclofen, a well characterized GABA$_B$ agonist, produced only a minor inhibition of Ca^{2+} channel current in neurons injected exclusively with GABA$_B$R1 cDNA, for greater than that (12.7 $\pm$ 3.2%) in control neurons injected with GFP cDNA (Couve et al., 1998). This lack of functional inhibition of Ca^{2+} currents in SCG is in agreement with the endoplasmic reticulum retention of GABA$_B$R1 after homomeric expression in neurons. In neurons injected with cDNAs for GABA$_B$R1a and GABA$_B$R2, bath application of 50 μM baclofen produced a substantial and reversible inhibition of Ca^{2+} channel current (recorded using Ba^{2+} as the charge carrier) (Fig. 1_A_). This inhibition was accompanied by a current-onset slowing similar to that reported after activation of many other endogenous or heterologously expressed G-protein-coupled receptors in SCG neurons (Hille, 1994; Ikeda et al., 1995; Filippov et al., 1998, 1999). Bath application of 1 μM CGP62349, a specific antagonist of GABA$_B$ receptors (Billinton et al., 1999), blocked the effect of baclofen (Fig. 1_C,D_). Interestingly, washing out of the agonist produced an initial over-recovery of the current followed by a slow return to the control level [as reported for some other receptors (see Meza et al., 1999)].

To study the requirement of heterodimer formation in the ability of recombinant GABA$_B$ receptors to couple to Ca^{2+} channels, GABA$_B$R2 cDNA, in the absence of GABA$_B$R1, was injected into SCG neurons. Surprisingly, Ca^{2+} channel currents were also inhibited in neurons injected exclusively with GABA$_B$R2. This effect was indistinguishable from that obtained after coexpression of GABA$_B$R1 and GABA$_B$R2 (Fig. 1_B_). Thus, the mean inhibition of Ca^{2+} channel current (measured 10 msec after onset of the test pulse) by 50 μM baclofen was 56.1 $\pm$ 3.2% for neurons expressing recombinant GABA$_B$R1 and GABA$_B$R2 and 52.3 $\pm$ 4.8% for neurons expressing recombinant GABA$_B$R2 exclusively (Fig. 1_D_).

GABA$_B$-mediated inhibition of Ca^{2+} current is strongly voltage dependent, suggesting involvement of the G-protein $\beta\gamma$ subunit

Slowing of the current onset by baclofen in neurons with heterologously expressed GABA$_B$R1 and GABA$_B$R2 or GABA$_B$R2 indicated that GABA$_B$ receptor-mediated inhibition of Ca^{2+} channel current was voltage dependent and relieved during depolarization (cf. Bean, 1989), as reported previously for other G-protein-coupled receptors. Indeed, a double-pulse protocol demonstrated that inhibition of Ca^{2+} current produced by baclofen was greatly reduced after a 120 mV depolarizing prepulse (Fig. 2_A_). The prepulse also abolished the slowing of the current onset produced by baclofen. Identical results were obtained with
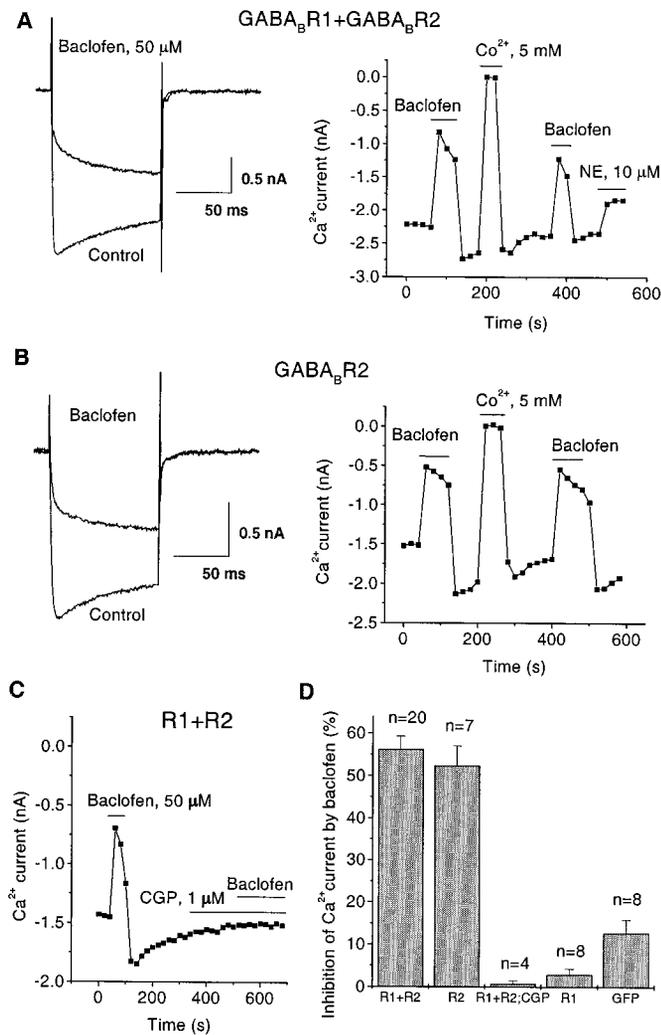
Figure 1. Coupling of heterologously expressed $GABA_BR1$ and $GABA_BR2$ receptors to N-type Ca^{2+} channels in sympathetic neurons. Currents were recorded by stepping for 100 msec every 20 sec from -90 to 0 mV and leak-corrected by subtracting currents remaining after substituting 5 mM Co^{2+} for Ba^{2+}. *A, Left, B, Left, Records* show superimposed leak-subtracted currents in the absence (*Control*) and presence of 50 μM baclofen. *A, Right, B, Right, C, Plots* show the time course of changes in current amplitude measured 10 msec from the onset of the test pulse. *Solid horizontal bars* indicate the time of exposure to baclofen, CGP62349 (*CGP*), Co^{2+}, or norepinephrine (*NE*). *D, Bar charts* show the mean inhibition of I_{Ba} amplitude by 50 μM baclofen in neurons injected with $GABA_B$ cDNAs + GFP cDNA or with GFP cDNA alone (*left*). Error bars show SEM (n = number of cells). Note that heterologously expressed $GABA_BR1$ + $GABA_BR2$ (*R1+R2*) or $GABA_BR2$ (*R2*) but not $GABA_BR1$ (*R1*) receptors couple to Ca^{2+} channels.
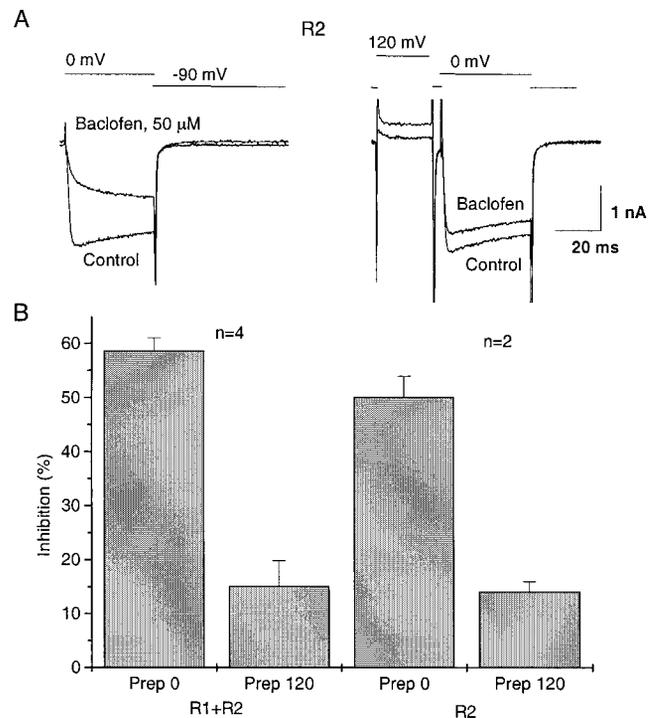

Figure 2. Voltage dependence of $GABA_B$-mediated inhibition of Ca^{2+} current. Neurons were injected with $GABA_BR1$ cDNA + $GABA_BR2$ cDNA (*R1+R2*) or with $GABA_BR2$ cDNA (*R2*). *A, Records* show superimposed Ca^{2+} currents obtained with a double-pulse voltage protocol (*top*) in the absence (*Control*) and presence of 50 μM baclofen. *B, Bar charts* show the mean percent current inhibition (measured after 10 msec at the 0 mV command potential) produced by baclofen before (*Prep 0*) and after (*Prep 120*) the +120 mV prepulse. Note that the prepulse almost abolished $GABA_B$-mediated inhibition of Ca^{2+} current and eliminated the slowing of the kinetics.

1995; Herlitze et al., 1996; Ikeda, 1996; Jones and Elmslie, 1997; Delmas et al., 1998a,b).

A PTX-sensitive G-protein mediates Ca²⁺ current inhibition after activation of recombinant GABA_BR1 and GABA_BR2 or GABA_BR2 receptors

A strong voltage dependency of Ca^{2+} channel current inhibition by baclofen in our experiments suggested that the recombinant $GABA_B$ receptors coupled to Ca^{2+} channels via PTX-sensitive G-proteins (for review, see Hille, 1994). Therefore, to determine the identity of the $G\alpha$-protein that mediates the Ca^{2+} current inhibition in SCG neurons, responses to baclofen were determined after pretreatment with PTX. Indeed, after PTX pretreatment (500 ng/ml), baclofen no longer inhibited Ca^{2+} channel currents in neurons expressing recombinant $GABA_BR1$ and $GABA_BR2$ receptors. PTX sensitivity was identical when cells expressing recombinant $GABA_BR2$ were studied. These observations are summarized in Figure 3B. Taken together they clearly indicate that PTX pretreatment prevents Ca^{2+} channel current inhibition in neurons expressing recombinant $GABA_BR1$ and $GABA_BR2$ or $GABA_BR2$ alone (Fig. 3). These results strongly suggest that only PTX-sensitive G-proteins mediate Ca^{2+} current inhibition after activation of recombinant $GABA_B$ receptors. The complete disappearance of the baclofen response in the presence of PTX was unique to $GABA_B$ receptors because the same concentration of PTX was not capable of totally abolishing the response of endogenous α-adrenergic receptors to norepinephrine (Fig. 3A). The small PTX-insensitive effect of norepi-

both heterologously expressed $GABA_BR1$ and $GABA_BR2$ or $GABA_BR2$ receptors. Inhibition was reduced from 58.7 ± 2.4 to $15.2 \pm 4.7\%$ in neurons with heterologously expressed $GABA_BR1$ and $GABA_BR2$ and from 50.2 ± 3.8 to $14.1 \pm 1.8\%$ in neurons expressing $GABA_BR2$ (Fig. 2B). A similar effect was reported for the modulation of Ca^{2+} current by endogenous $GABA_B$ receptors in sensory neurons and in calyx nerve terminals of the mammalian auditory brainstem (Grassi and Lux, 1989; Isaacson, 1998). Similar voltage-dependent effects have been generally interpreted as produced by a direct interaction of G-protein $\beta\gamma$ subunits with an α subunit of the Ca^{2+} channel (Dolphin,
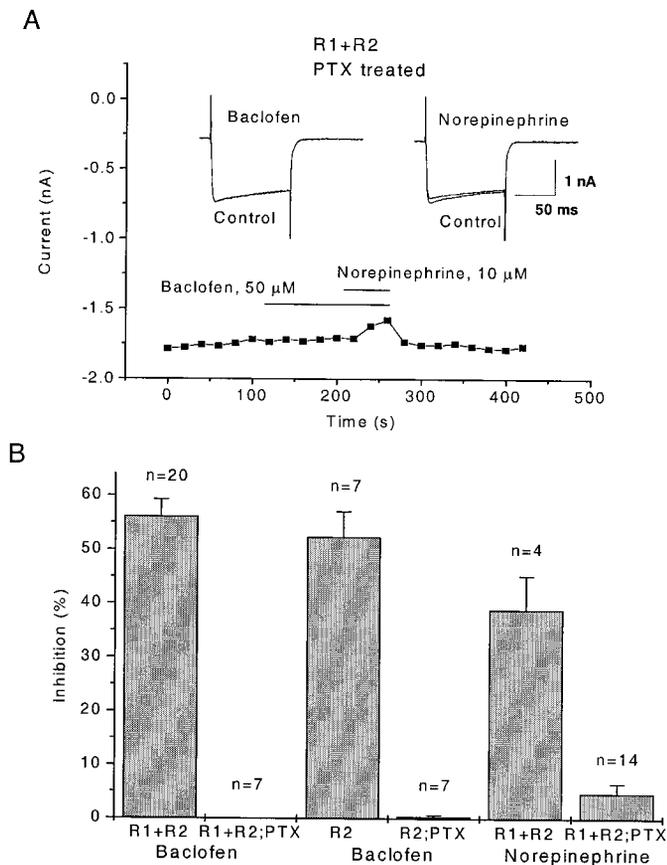
Figure 3. Recombinant GABA$_B$ receptor couples to Ca^{2+} channels only via PTX-sensitive G-proteins. *A, Top, Records* show superimposed leak-subtracted currents in the absence (*Control*) and presence of 50 μM baclofen (*left*) or 10 μM norepinephrine (*right*) in neurons pretreated with PTX (0.5 μg/ml; overnight). *Bottom,* A time *plot* of I_{Ba} amplitude is shown. *B,* Mean inhibition of I_{Ba} amplitude by 50 μM baclofen or 10 μM norepinephrine in neurons pretreated with PTX and in untreated neurons is shown. Note that PTX pretreatment completely prevented GABA$_B$R1 + GABA$_B$R2 (*R1+R2*)- or GABA$_B$R2 (*R2*)-mediated inhibition of Ca^{2+} channel current, whereas a small effect of norepinephrine remained.

nephrine is in agreement with a minor involvement of PTX-insensitive G-proteins in adrenergic receptor-mediated function (Hille, 1994; Delmas et al., 1999).

Heteromeric assembly of GABA$_B$R1 with GABA$_B$R2 is required to couple effectively to Ca^{2+} channels in neurons

The results presented above showing the strong inhibition of Ca^{2+} channel currents by GABA$_B$R2 cDNA alone were of interest because heterologous expression of GABA$_B$R2 is not sufficient to produce functional coupling to adenylyl cyclase or inward rectifier potassium channels in *Xenopus* oocytes or HEK293 cells (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). One possible explanation is that SCG neurons express endogenous GABA$_B$R1 subunit that is not functionally coupled to Ca^{2+} channels but that, on expression of GABA$_B$R2, coassembles with recombinant GABA$_B$R2 to constitute a functional heteromeric receptor indistinguishable from a recombinant dimer. Several experiments were designed to test this hypothesis.

First, the expression of GABA$_B$R1 and GABA$_B$R2 was analyzed in SCG neurons via immunoblotting with specific antibodies against these proteins. A specific antibody was raised against
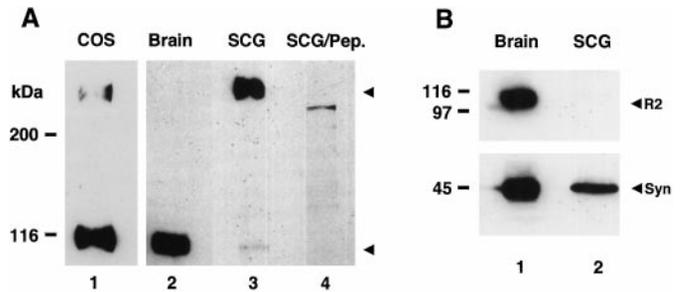

Figure 4. GABA$_B$R1 but not GABA$_B$R2 receptors are present in SCG neurons. *A,* Cell extracts derived from SCG neurons and control preparations were immunoblotted with GABA$_B$R1a antibodies. *Lane 1,* Extracts derived from COS cells transfected with GABA$_B$R1a; *lane 2,* crude brain membranes; *lane 3,* extracts derived from superior cervical ganglia; *lane 4,* same as in *lane 3* but with the antibody preincubated with 25 μg/ml GABA$_B$R1a peptide CHPPWEGGIRYRGLTRDQVK. Relevant molecular weight markers are indicated on the *left*. *B,* Cell extracts derived from SCG neurons and control preparations were immunoblotted with GABA$_B$R2 antibodies (*R2; top*) or synaptophysin (*Syn; bottom*). *Lane 1,* Crude brain membranes; *lane 2,* extracts derived from superior cervical ganglia. Relevant molecular weight markers are indicated on the *left*. *Pep,* Peptide.

a peptide in the N-terminal domain of the GABA$_B$R1a receptor, and expression of recombinant GABA$_B$R1 was used to test the specificity of this antisera. In agreement with previous observations, the antibody recognized GABA$_B$R1a as two specific bands in cell lysates derived from transfected COS cells. A lower band of ~120 kDa represents the monomeric receptor, and an ~250 kDa band presumably corresponds to receptor aggregates (Fig. 4*A, lane 1*). In contrast, a single ~120 kDa band is detected in crude brain membranes in agreement with previous observations (Fig. 4*A, lane 2*) (Benke et al., 1999). Interestingly, low levels of an ~120 kDa band corresponding to the GABA$_B$R1a protein were detected in SCG extracts. Unexpectedly, an aggregate form of the GABA$_B$R1a protein was predominant in these cell lysates (Fig. 4*A, lane 3*). This band matches the ~250 kDa form of GABA$_B$R1a observed in transfected COS cells. The specificity of the GABA$_B$R1a antibody was demonstrated by competition with a GABA$_B$R1 peptide before immunoblotting (Fig. 4*A, lane 4*).

Second, to determine the expression of GABA$_B$R2 in SCG, immunoblots with antibodies against GABA$_B$R2 were performed. A single band of ~110 kDa was detected in crude brain membranes (Fig. 4*B, top*). SCG extracts showed no detectable levels of GABA$_B$R2 (Fig. 4*B, top*). An immunoblot with anti-synaptophysin antibodies indicates that protein levels and the integrity of cell extracts were similar between brain and SCG preparations (Fig. 4*B, bottom*). These observations were confirmed by detection of GABA$_B$R1 (see Fig. 6) but not GABA$_B$R2 (data not shown) by immunofluorescence in SCG neurons.

To investigate further the inhibition of Ca^{2+} current by GABA$_B$ receptor subunits in neurons, the effects of baclofen on expressed GABA$_B$R1 and GABA$_B$R2 or GABA$_B$R2 receptors were studied in detail using full dose–response curves. Baclofen inhibited Ca^{2+} currents more effectively and with higher potency in cells expressing GABA$_B$R1 and GABA$_B$R2 receptors than in cells expressing only heterologous GABA$_B$R2 receptors (Fig. 5*A*). The IC$_{50}$ for inhibition was 0.09 ± 0.01 μM for GABA$_B$R1 and GABA$_B$R2 and approximately four times higher (0.38 ± 0.05 μM) for GABA$_B$R2. To confirm these observations using a different methodology, SCG neurons were injected either with GABA$_B$R2 cDNA or with an equal mixture of GABA$_B$R2 cDNA
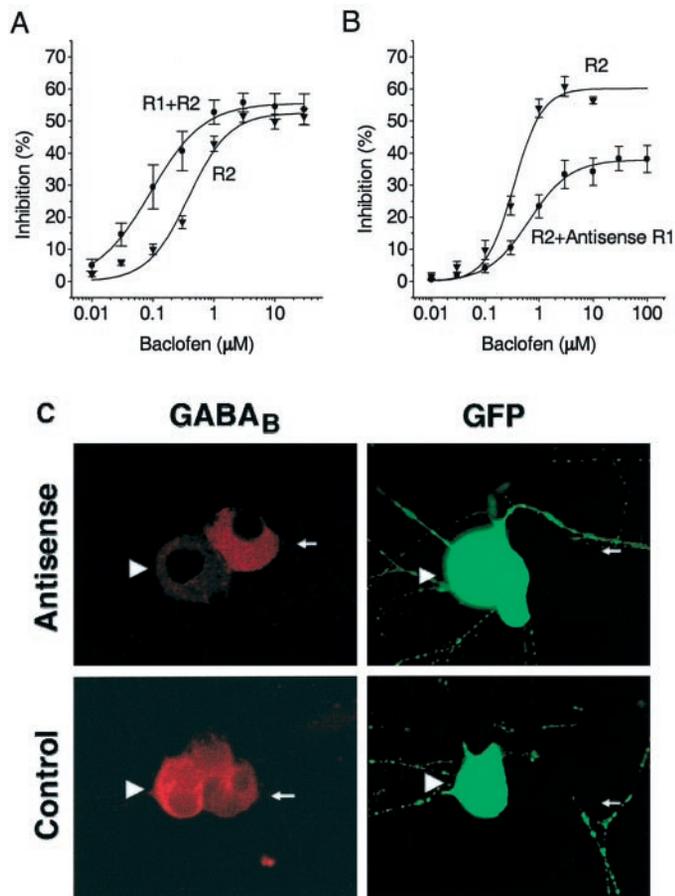
Figure 5. Heteromeric assembly of GABA$_B$R1 with GABA$_B$R2 is required to couple effectively to Ca^{2+} channels in neurons. *A, B, Plots* show the concentration dependence of Ca^{2+} current inhibition by baclofen in SCG neurons injected with GABA$_B$R1 cDNA + GABA$_B$R2 cDNA (*R1+R2*) or GABA$_B$R2 cDNA (*R2*) ~16–20 hr before recordings (*A*) or with GABA$_B$R2 cDNA (*R2*) or GABA$_B$R2 cDNA + GABA$_B$R1 antisense cDNA (*R2+antisense R1*) ~40 hr before recordings (*B*). *Curves* were fitted to pooled data points (mean ± SEM) using Origin 4.1 software to the Hill equation: $y = y_{max}x^{nH}/(x^{nH} + K^{nH})$, where y is the observed percent inhibition, y_{max} is the extrapolated maximal percent inhibition, x is the baclofen concentration (micromolar), K is the IC$_{50}$ (micromolar), and n_H is the Hill coefficient. *A,* For *R1+R2*, IC$_{50}$ = 0.09 ± 0.01 μM; Hill coefficient = 1.00 ± 0.13; and maximal inhibition = 55.55 ± 1.68%. For *R2*, IC$_{50}$ = 0.38 ± 0.05 μM; Hill coefficient = 1.30 ± 0.20; and maximal inhibition = 52.55 ± 1.90%. *B,* For *R2*, IC$_{50}$ = 0.34 ± 0.04 μM; Hill coefficient = 1.66 ± 0.28; and maximal inhibition = 60.14 ± 2.30%. For *R2+antisense R1*, IC$_{50}$ = 0.66 ± 0.15 μM; Hill coefficient = 1.14 ± 0.24; and maximal inhibition = 37.93 ± 1.92%. Note that heterologously coexpressed GABA$_B$R1 and GABA$_B$R2 inhibit Ca^{2+} current more effectively than does heterologously expressed GABA$_B$R2 and that reduction of endogenous GABA$_B$R1 expression by antisense cDNA significantly reduces inhibition of Ca^{2+} current. *C,* Immunofluorescence with GABA$_B$R1 antibodies in cells injected with GABA$_B$R1 cDNA antisense is shown. Cells were injected with either GFP (*bottom panels)* or GFP plus GABA$_B$R1a antisense (*top panels*) cDNA. *Right panels* show GFP fluorescence of injected neurons. *Left panels* show the same neurons stained for GABA$_B$R1. In both cases the *leftmost* cell has been injected (*arrowheads*), whereas the *rightmost* cell has not (*arrows*). Only cells injected with GABA$_B$R1 antisense cDNA show a decrease in GABA$_B$R1 expression when compared with noninjected cells.

and GABA$_B$R1 antisense cDNA to reduce the expression of endogenous GABA$_B$R1 protein. As expected, immunofluorescence with GABA$_B$R1 antibodies revealed detectable levels of GABA$_B$R1 in all sympathetic neurons, and levels were reduced in

cells injected with GFP and GABA$_B$R1 antisense cDNA (Fig. 5*C*, *top left*, *arrowhead*). Furthermore, coexpression of the sense and antisense cDNAs of GABA$_B$R1 resulted in a significant decrease in expression of recombinant GABA$_B$R1 protein (data not shown). Control cells injected with GFP alone showed no difference in expression of GABA$_B$R1 (Fig. 5*C*, *bottom left*, *arrowhead*). In accordance with these observations, inhibition of Ca^{2+} channel currents in neurons injected with GABA$_B$R1 antisense cDNA was significantly lower than inhibition in control neurons for all effective doses of baclofen (Fig. 5*B*).

Taken together, the results presented above indicate that SCG neurons contain detectable levels of GABA$_B$R1a and suggest that responses to baclofen in neurons injected with GABA$_B$R2 result from activation of a heterodimer formed between endogenous GABA$_B$R1a and recombinant GABA$_B$R2 receptors. Interestingly, trace levels of GABA$_B$R2 were observed occasionally in SCG extracts. This might generate trace levels of endogenous GABA$_B$R1/R2 dimer and hence provide an explanation for the low response of control SCG neurons to baclofen (Fig. 1*D*).

Endogenous GABA$_B$R1 is predominantly localized to the endoplasmic reticulum in SCG neurons

As reported previously, recombinant GABA$_B$R1 receptors are retained in an intracellular compartment when expressed in neuronal and non-neuronal cell types (Couve et al., 1998). To determine whether endogenous GABA$_B$R1 receptors failed to reach the cell surface in SCG neurons, immunofluorescence with GABA$_B$R1 antibodies was performed. The GABA$_B$R1 receptor was found mainly restricted to the cell body of SCG neurons (Fig. 6*A, left*). Detection of MAP2, a somatodendritic marker, shows a different distribution pattern with predominant staining in cell projections (Fig. 6*A*). The pattern difference is still apparent after the levels of fluorescence have been equalized.

To determine the subcellular distribution of GABA$_B$R1, SCG neurons were stained for GABA$_B$R1 and DPI, an endoplasmic reticulum marker. DPI is present in neuronal and non-neuronal cell types from the SCG preparation (Fig. 6*B*). In contrast, GABA$_B$R1 is present exclusively in neurons, and it overlaps almost entirely with DPI in these cells. These observations strongly suggest that both proteins colocalize in the endoplasmic reticulum.

GABA$_B$R1 is localized to the plasma membrane after coexpression of GABA$_B$R2

To provide additional evidence of the formation of functional, cell surface heterodimers after expression of GABA$_B$R2, SCG neurons were microinjected with GABA$_B$R1 or GABA$_B$R2 independently or with GABA$_B$R1 and GABA$_B$R2 simultaneously. As reported previously (Couve et al., 1998), a myc-tagged version of GABA$_B$R1 is retained intracellularly in SCG neurons (Fig. 7, *top*). In contrast, a FLAG-tagged version of GABA$_B$R2 is targeted efficiently to the cell surface (Fig. 7, *middle*). Consistent with our electrophysiological findings, expression of GABA$_B$R2 changes the subcellular distribution of GABA$_B$R1 from the endoplasmic reticulum to the plasma membrane (Fig. 7, *bottom*). These observations support the idea that GABA$_B$R2 rescues GABA$_B$R1 from intracellular compartments and strongly suggest that heterodimer formation is a requirement for GABA$_B$ receptor function.

DISCUSSION

In this report we demonstrate that coexpressed GABA$_B$R1 and GABA$_B$R2 subunits produce a functional receptor in sympa-
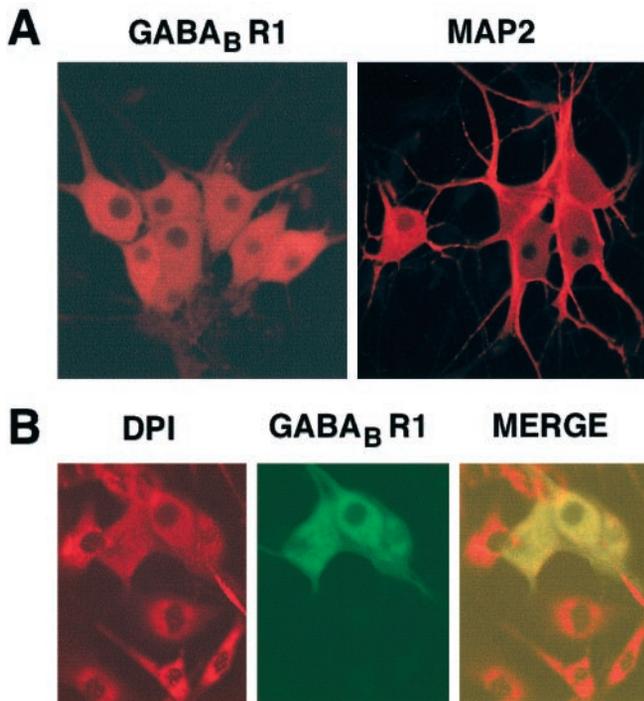
Figure 6. GABA_BR1 is predominantly localized to the endoplasmic reticulum in SCG neurons. *A,* Expression and distribution of GABA_BR1 in SCG neurons were examined after staining with a GABA_BR1 antibody directed against the C-terminal domain of GABA_BR1 (*left*) or with antibodies directed against MAP2 (*right*). The levels of fluorescence were equalized to compare distribution of the two proteins. *B,* Subcellular distribution of GABA_BR1 in SCG neurons was analyzed by coimmunofluorescence with DPI (*left*) and GABA_BR1 (*middle*) antibodies. DPI staining is not restricted to neurons and overlaps almost completely with GABA_BR1 staining in SCGs (merged image; *right*).

Figure 7. Recombinant GABA_BR1 is expressed at the cell surface after coexpression of GABA_BR2 in SCG neurons. Expression and distribution of GABA_BR1 tagged at the N-terminal domain with the myc epitope (*R1Myc; top*) and GABA_BR2 tagged at the N-terminal domain with the FLAG epitope (*R2Flag; middle*) were examined after staining with 9E10 and FLAG antibodies, respectively. The distribution of *R1Myc* after coexpression of GABA_BR2 (*R2*) was analyzed by immunofluorescence with 9E10 antibodies (*bottom*). On the *right* are merged images of intracellular and cell surface staining. The *R1Myc*-staining pattern changes dramatically after injection of GABA_BR2.

thetic neurons that couples effectively to N-type Ca²⁺ channels. Thus, heteromeric assembly of GABA_BR1 and GABA_BR2 receptor subunits appears to be necessary for coupling to all three known effectors: Ca²⁺ channels, adenylyl cyclase, and inward rectifier K⁺ channels (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). It might be noted, however, that the IC_{50} for inhibition of the Ca²⁺ current in sympathetic neurons (~90 nM) was appreciably lower than that required to inhibit GIRK channels in non-neuronal cells, suggesting that expression of GABA_B receptor dimers in their natural (neuronal) environment allows more effective ion channel coupling.

To the degree that the present experiments allow, the mechanism by which these exogenously expressed receptors inhibit neuronal Ca²⁺ channels appears identical to that used by native GABA_B receptors present in other neurons. Thus, inhibition was strongly reduced by *pertussis* toxin, implying mediation by members of the G_i/G_o family of G-proteins [probably G_o (see Campbell et al., 1993; Menon-Johansson et al., 1993)]. Also, the strong voltage dependence of inhibition in sympathetic neurons accords with previous observations on sensory neurons (Grassi and Lux, 1989), suggesting that it was mediated by βγ subunits of the G-protein (Dolphin, 1995; Jones and Elmslie, 1997). Thus, our observations on the expression and functional coupling of recombinant GABA_B receptors in sympathetic neurons are likely to be relevant to the properties of natively expressed receptors.

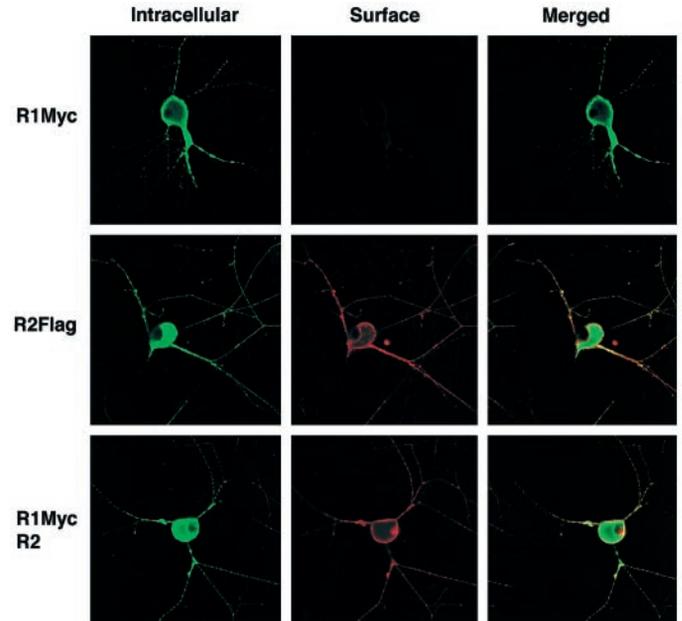Immunoblot analysis demonstrated that the predominant form of GABA_BR1 in SCG corresponds to a large ~250 kDa protein band. The nature of this high-molecular weight form of GABA_BR1 is not known, but it is unlikely to correspond to receptor dimers because homomeric interactions have not been shown to occur (Fritschy et al., 1999). A similar protein band is normally observed in other heterologous expression systems in which the GABA_BR1 receptor is not functional (Couve et al., 1998). We hypothesize that this structure corresponds to nonfunctional GABA_BR1 receptor aggregates retained in the endoplasmic reticulum.

In this context, our experiments strongly suggest that GABA_BR2 forms an essential subunit of the functional receptor and that it is required both for assembly and membrane insertion of the receptor. Thus, functional membrane receptors were generated not only by coexpression of recombinant GABA_BR1 and GABA_BR2 plasmids but also on expression of exogenous GABA_BR2 subunits alone. We attribute this to coassembly with the native GABA_BR1 subunits normally present in these sympathetic neurons, because reduction of GABA_BR1 protein by antisense expression also reduced the response of GABA_BR2-expressing cells to baclofen (Fig. 5). Because GABA_BR1 protein was predominantly confined to the endoplasmic reticulum, this also suggests that GABA_BR2 plays a crucial role in trafficking and membrane insertion of the dimeric receptor. The fact that GABA_BR2 is localized to the plasma membrane and traffics GABA_BR1 in heterologous cells (White et al., 1998; Martin et al., 1999) and in SCG neurons supports these observations.

Cultured SCG neurons respond poorly to GABA_B agonists and are generally thought to be devoid of GABA_B receptors. However, GABA_B responses may appear as a result of GABA_BR2 expression during pathology, in certain stages of development, or

under specific and local stimuli. Our findings together with the fact that GABA$_B$ agonists have been shown to modify superior cervical ganglia after prolonged applications (Parducz et al., 1990), that SCG neurons contain GABA$_A$ receptors (Brown et al., 1979), and that SCG neurons are innervated by GABAergic terminals (Kasa et al., 1988) certainly suggest that GABA$_B$ receptors have the potential to function in sympathetic neurons. Nevertheless, the conditions under which GABA$_B$ receptors may be physiologically expressed remain to be determined. If this hypothesis is correct, other neuronal types might use a similar regulatory mechanism, in which expression of the GABA$_B$R2 subunit or other accessory protein determines the formation of functional receptors by trafficking existent GABA$_B$R1 from the endoplasmic reticulum to the plasma membrane.

In situ hybridization studies have revealed certain areas in the CNS where expression of GABA$_B$R1 is much greater than GABA$_B$R2 and others where GABA$_B$R1 is present and GABA$_B$R2 is not (Jones et al., 1998; Kaupmann et al., 1998; Martin et al., 1999). These observations have led to the suggestion that GABA$_B$R1 can function as a monomer or with accessory proteins other than GABA$_B$R2 in some areas of the CNS (Marshall et al., 1999). The fact that baclofen produced a small inhibition of the Ca^{2+} current in sympathetic neurons in the absence of exogenous GABA$_B$R2 subunits might appear to offer some support for this view. However, because trace levels of endogenous GABA$_B$R2 protein were occasionally detected in SCG extracts, we cannot exclude the possibility that the small effects of baclofen on uninjected cells might have resulted from activation of a small number of endogenous GABA$_B$R1/R2 dimers.

The question also arises whether GABA$_B$R2 subunits might form functional receptors independent of GABA$_B$R1 subunits. GABA$_B$R2 has been reported to couple negatively to adenylyl cyclase in transfected COS cells (Martin et al., 1999) and HEK293 cells (Kuner et al., 1999). However, the effectiveness of this coupling is under question because the baclofen concentrations used in these experiments were very high (300–500 μM; compared, for example, with an IC$_{50}$ of ~90 nM for Ca^{2+} current inhibition mediated by GABA$_B$R1 and GABA$_B$R2 in the present experiments), and GABA$_B$R2 does not couple to the inward rectifier GIRK channels coexpressed in *Xenopus* oocytes or HEK293 cells (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). Our experiments with GABA$_B$R1 antisense cDNA injections in SCG neurons confirm that responses apparently mediated by exogenous GABA$_B$R2 receptors are primarily (and possibly entirely) caused by formation of GABA$_B$R1/GABA$_B$R2 dimers with endogenous GABA$_B$R1 subunits. Nevertheless, more conclusive evidence is necessary to exclude the possibility that either GABA$_B$R1 or GABA$_B$R2 might be able to function independently. New subunit-specific agonists and definitive binding studies are necessary to resolve this issue.

Notwithstanding such considerations, the principal point emerging from the present experiments is that efficient coupling of GABA$_B$ receptors to Ca^{2+} channels in sympathetic neurons requires the heteromeric assembly of both GABA$_B$R1 and GABA$_B$R2 subunits and therefore that this is likely to be true for those endogenous GABA$_B$ receptors responsible for Ca^{2+} current inhibition (and resultant presynaptic inhibition) in other parts of the mammalian nervous system.

REFERENCES

Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. Nature 340:153–156.

Benke D, Honer M, Michel C, Bettler B, Mohler H (1999) Gamma-aminobutyric acid type B receptor splice variant proteins GBR1a and GBR1b are both associated with GBR2 in situ and display differential regional and subcellular distribution. J Biol Chem 274:27323–27330.

Billinton A, Upton N, Bowery NG (1999) GABA$_B$ receptor isoforms GBR1a and GBR1b appear to be associated with pre- and post-synaptic elements respectively in rat and human cerebellum. Br J Pharmacol 126:1387–1392.

Bittiger H, Froestl W, Mickel S, Olpe HR (1993) GABA$_B$ receptor antagonists: from synthesis to therapeutic applications. Trends Pharmacol Sci 14:391–394.

Bowery NG (1993) GABA$_B$ receptor pharmacology. Annu Rev Pharmacol Toxicol 33:109–147.

Brown DA, Adams PR, Higgins AJ, Marsh S (1979) Distribution of gaba-receptors and gaba-carriers in the mammalian nervous system. J Physiol (Paris) 75:667–671.

Campbell V, Berrow N, Dolphin AC (1993) GABAB receptor modulation of Ca2+ currents in rat sensory neurones by the G protein G(0): antisense oligonucleotide studies. J Physiol (Lond) 470:1–11.

Caulfield MP, Jones S, Vallis Y, Buckley NJ, Kim G-D, Milligan G, Brown DA (1994) Muscarinic M-current inhibition via G$_{\alpha q/11}$ and α-adrenoceptor inhibition of Ca^{2+} current via G$_{\alpha o}$ in rat sympathetic neurones. J Physiol (Lond) 477:415–422.

Couve A, Filippov AK, Connolly CN, Bettler B, Brown DA, Moss SJ (1998) Intracellular retention of recombinant GABA$_B$ receptors. J Biol Chem 273:26361–26367.

Davies CH, Starkey SJ, Pozza MF, Collingridge GL (1991) GABA autoreceptors regulate the induction of LTP. Nature 349:609–611.

Delmas P, Brown DA, Dayrell M, Abogadie FC, Caulfield MP, Buckley NJ (1998a) On the role of endogenous G-protein $\beta\gamma$ subunits in N-type Ca^{2+} current inhibition by neurotransmitters in rat sympathetic neurones. J Physiol (Lond) 506:319–329.

Delmas P, Abogadie FC, Dayrell M, Haley JE, Milligan G, Caulfield MP, Brown DA, Buckley NJ (1998b) G-proteins and G-protein subunits mediating cholinergic inhibition of N-type calcium currents in sympathetic neurones. Eur J Neurosci 10:1654–1666.

Delmas P, Abogadie FC, Milligan G, Buckley NJ, Brown DA (1999) $\beta\gamma$ dimers derived from G$_o$ and G$_i$ proteins contribute different components of adrenergic inhibition of Ca^{2+} channels in rat sympathetic neurones. J Physiol (Lond) 518:23–36.

Dichter MA (1997) Basic mechanisms of epilepsy: targets for therapeutic intervention. Epilepsia 38[Suppl 9]:2–6.

Dolphin AC (1995) Voltage-dependent calcium channels and their modulation by neurotransmitters and G proteins. Exp Physiol 80:1–36.

Dolphin AC, Scott RH (1987) Calcium channel currents and their inhibition by (−)-baclofen in rat sensory neurones: modulation by guanine nucleotides. J Physiol (Lond) 386:1–17.

Filippov AK, Webb TE, Barnard EA, Brown DA (1997) Inhibition by heterologously-expressed P2Y$_2$ nucleotide receptors of N-type calcium currents in rat sympathetic neurones. Br J Pharmacol 121:849–851.

Filippov AK, Webb TE, Barnard EA, Brown DA (1998) P2Y$_2$ nucleotide receptors expressed heterologously in sympathetic neurons inhibit both N-type Ca^{2+} and M-type K$^+$ currents. J Neurosci 18:5170–5179.

Filippov AK, Webb TE, Barnard EA, Brown DA (1999) Dual coupling of heterologously-expressed rat P2Y$_6$ nucleotide receptors to N-type Ca^{2+} and M-type K$^+$ currents in rat sympathetic neurons. Br J Pharmacol 126:1009–1017.

Fritschy JM, Meskenaite V, Weinmann O, Honer M, Benke D, Mohler H (1999) GABAB-receptor splice variants GB1a and GB1b in rat brain: developmental regulation, cellular distribution and extrasynaptic localization. Eur J Neurosci 11:761–768.

Grassi F, Lux HD (1989) Voltage-dependent GABA-induced modulation of Ca^{2+} channels in chick sensory neurons. Neurosci Lett 105:113–119.

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA (1996) Modulation of Ca^{2+} channels by G-protein $\beta\gamma$ subunits. Nature 105:113–119.

Hille B (1994) Modulation of ion-channel function by G-protein-coupled receptors. Trends Neurosci 17:531–536.

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$ subunits. Nature 380:255–258.

Ikeda SR, Lovinger DM, McCool BA, Lewis DL (1995) Heterologous

expression of metabotropic glutamate receptors in adult rat sympathetic neurons: subtype-specific coupling to ion channels. Neuron 14:1029–1038.

Isaacson JS (1998) GABA$_B$ receptor-mediated modulation of presynaptic currents and excitatory transmission at a fast central synapse. J Neurophysiol 80:1571–1576.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao WJ, Johnson M, Gunwaldsen C, Huang LY, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C (1998) GABA$_B$ receptors function as a heteromeric assembly of the subunits GABA$_B$R1 and GABA$_B$R2. Nature 396:674–679.

Jones SW, Elmslie KS (1997) Transmitter modulation of neuronal calcium channels. J Membr Biol 155:1–10.

Kasa P, Joo F, Dobo E, Wenthold RJ, Ottersen OP, Storm-Mathisen J, Wolff JR (1988) Heterogeneous distribution of GABA-immunoreactive nerve fibers and axon terminals in the superior cervical ganglion of adult rat. Neuroscience 26:635–644.

Kaupmann K, Huggel K, Heid J, Flor PJ, Bischoff S, Mickel SJ, McMaster G, Angst C, Bittiger H, Froestl W, Bettler B (1997) Expression cloning of GABA$_B$ receptors uncovers similarity to metabotropic glutamate receptors. Nature 386:239–246.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbacher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B (1998) GABA$_B$-receptor subtypes assemble into functional heteromeric complexes. Nature 396:683–687.

Kerr DI, Ong J (1995) GABA$_B$ receptors. Pharmacol Ther 67:187–246.

Kuner R, Kohr G, Grunewald S, Eisenhardt G, Bach A, Kornau HC (1999) Role of heteromer formation in GABA$_B$ receptor function. Science 283:74–77.

Marshall FH, Jones KA, Kaupmann K, Bettler B (1999) GABA$_B$ receptors—the first 7TM heterodimers. Trends Pharmacol Sci 20:396–399.

Martin SC, Russek SJ, Farb DH (1999) Molecular identification of the human GABA$_B$R2: cell surface expression and coupling to adenylyl cyclase in the absence of GABA$_B$R1. Mol Cell Neurosci 13:180–191.

Menon-Johansson AS, Berrow N, Dolphin AC (1993) G(o) transduces GABA$_B$-receptor modulation of N-type calcium channels in cultured dorsal root ganglion neurons. Pflügers Arch 425:335–343.

Meza U, Bannister R, Melliti K, Adams BJ (1999) Biphasic, opposing modulation of cloned neuronal alpha1E Ca channels by distinct signaling pathways coupled to M2 muscarinic acetylcholine receptors. J Neurosci 19:6806–6817.

Mott DD, Lewis DV (1994) The pharmacology and function of central GABA$_B$ receptors. Int Rev Neurobiol 36:97–223.

Olpe HR, Worner W, Ferrat T (1993) Stimulation parameters determine role of GABAB receptors in long-term potentiation. Experientia 49:542–546.

Parducz A, Joo F, Siklos L, Wolff JR (1990) Fine structural changes in the superior cervical ganglion of adult rats after long-term administration of baclofen, a GABA$_B$ receptor agonist. Neuroscience 36:239–245.

Takahashi T, Kajikawa Y, Tsujimoto T (1998) G-Protein-coupled modulation of presynaptic calcium currents and transmitter release by a GABA$_B$ receptor. J Neurosci 18:3138–3146.

White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH (1998) Heterodimerization is required for the formation of a functional GABA$_B$ receptor. Nature 396:679–682.