0026-895X/01/5901-144–152$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 59:144–152, 2001

Vol. 59, No. 1
142/875313
Printed in U.S.A.

γ-Aminobutyric Acid Type B Receptors with Specific Heterodimer Composition and Postsynaptic Actions in Hippocampal Neurons Are Targets of Anticonvulsant Gabapentin Action

GORDON Y. K. NG, SANDRINE BERTRAND, RICHARD SULLIVAN, NATHALIE ETHIER, JENNIFER WANG, JIM YERGEY, MICHEL BELLEY, LAIRD TRIMBLE, KEVIN BATEMAN, LUANDA ALDER, ALISON SMITH, RUTH MCKERNAN, KATHLEEN METTERS, GARY P. O'NEILL, JEAN-CLAUDE LACAILLE, and TERENCE E. HÉBERT

Merck Frosst Center for Therapeutic Research, Kirkland, Canada (G.Y.K.N., R.S., J.W., J.Y., M.B., L.T., K.B., K.M., G.P.O.); Centre de recherche en sciences neurologiques et Département de physiologie, Université de Montréal, Montréal, Canada (S.B., J.-C.L.); Institut de Cardiologie de Montréal et le Groupe de Recherche sur le Système Nerveux Autonome, Université de Montréal, Montréal, Canada (N.E., T.E.H.); and Merck Sharp & Dohme Research Laboratories, Terlings Park, Harlow, Essex CM20 2QR, UK (L.A., A.S., R.M.)

Received March 10, 2000; received September 14, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

γ-Aminobutyric acid (GABA) activates two qualitatively different inhibitory mechanisms through ionotropic $GABA_A$ multisubunit chloride channel receptors and metabotropic $GABA_B$ G protein-coupled receptors. Evidence suggests that pharmacologically distinct $GABA_B$ receptor subtypes mediate presynaptic inhibition of neurotransmitter release by reducing Ca^{2+} conductance, and postsynaptic inhibition of neuronal excitability by activating inwardly rectifying K^+ (Kir) conductance. However, the cloning of $GABA_B$ gb1 and gb2 receptor genes and identification of the functional $GABA_B$ gb1-gb2 receptor heterodimer have so far failed to substantiate the existence of pharmacologically distinct receptor subtypes. The anticonvulsant, antihyperalgesic, and anxiolytic agent gabapentin (Neurontin) is a 3-alkylated GABA analog with an unknown mechanism of action. Here we report that gabapentin is an agonist at the $GABA_B$ gb1a-gb2 heterodimer coupled to Kir 3.1/3.2 inwardly rectifying K^+ channels in *Xenopus laevis* oocytes. Gabapentin was practically inactive at the human gb1b-gb2 heterodimer, a novel human gb1c-gb2 heterodimer and did not block GABA agonism at these heterodimer subtypes. Gabapentin was not an agonist at recombinant $GABA_A$ receptors as well. In CA1 pyramidal neurons of rat hippocampal slices, gabapentin activated postsynaptic K^+ currents, probably via the gb1a-gb2 heterodimer coupled to inward rectifiers, but did not presynaptically depress monosynaptic $GABA_A$ inhibitory postsynaptic currents. Gabapentin is the first $GABA_B$ receptor subtype-selective agonist identified providing proof of pharmacologically and physiologically distinct receptor subtypes. This selective agonism of postsynaptic $GABA_B$ receptor subtypes by gabapentin in hippocampal neurons may be its key therapeutic advantage as an anticonvulsant.

γ-Aminobutyric acid is an inhibitory amino acid neurotransmitter agonist at ionotropic $GABA_A/GABA_C$ multisubunit receptors and metabotropic $GABA_B$ G protein-coupled receptors (reviewed by Misgeld et al., 1995). $GABA_B$ receptors have been implicated in hippocampal long-term potentiation, slow-wave sleep, absence epilepsy, muscle relaxation, and antinociception (reviewed by Kerr and Ong, 1995). Many of the physiological roles of $GABA_B$ receptors can be attributed to the modulation of G protein-gated Ca^{2+} and K^+ channels (for review, see Kerr and Ong, 1995; Misgeld et al., 1995; Bowery and Enna, 2000). Presynaptic receptor activation has generally been reported to result in the inhibition of Ca^{2+} conductance presumably at P/Q and N-type Ca^{2+} channels leading to a decrease in the evoked release of neurotransmitters (Doze et al., 1995; Wu and Saggau, 1997). Postsynaptic receptor activation has generally been associated with increased K^+ currents, which result in membrane

The work in the laboratory of T.E.H. was supported by the Medical Research Council of Canada, the Heart and Stroke Foundation of Canada, and the Fonds de la Recherche en Santé du Québec (FRSQ). The work in the laboratory of J.-C.L. was supported by the Medical Research Council of Canada, the FRSQ, a Research Center grant from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) to the Groupe de Recherche sur le Système Nerveux Central (GRSNC), and an Équipe de Recherche grant from the FCAR. S.B. was supported by a postdoctoral fellowship from the GRSNC (FCAR).

ABBREVIATIONS: GABA, γ-aminobutyric acid; gb1, γ-aminobutyric acid $_B$R1 receptor subunit; gb2, γ-aminobutyric acid $_B$R2 receptor subunit; Kir, inwardly rectifying potassium (K^+) channel; CNS, central nervous system; ACSF, artificial cerebrospinal fluid; I-V, current-voltage; IPSC, inhibitory postsynaptic current; CNQX, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline; NMDA, *N*-methyl-D-aspartate; GPCR, G protein-coupled receptors.

hyperpolarization and inhibition of neuronal excitability. Studies in native tissues suggest the existence of high- and low-affinity $GABA_B$ receptors and pharmacologically distinct receptor subtypes that mediate pre- and postsynaptic actions, but proof is lacking [see Bowery and Enna (2000) and references therein].

Cloning has identified two major human $GABA_B$ receptor genes termed gb1 and gb2 (Kaupmann et al., 1997, 1998b; Jones et al., 1998; White et al., 1998; Kuner et al., 1999; Martin et al., 1999; Ng et al., 1999a,b). The human gb1 receptor gene encodes two structurally distinct N-terminal variants termed gb1a and gb1b, whereas the human gb2 receptor gene encodes a single form of the receptor. It is generally accepted that the functional $GABA_B$ receptor results from the coexpression and heterodimerization of gb1 and gb2 (Jones et al., 1998; Kaupmann et al., 1998b; White et al., 1998; Kuner et al., 1999; Ng et al., 1999a). Thus gb1 and gb2 may be more appropriately considered receptor subunits, but several studies have reported that gb1 and gb2 homomers are active (Kaupmann et al., 1997, 1998a; Kuner et al., 1999; Martin et al., 1999). Cloning, however, which has revealed structurally distinct $GABA_B$ receptors, has not substantiated the existence of pharmacologically distinct receptor subtypes.

Gabapentin (Neurontin; 1-(aminomethyl)cyclohexaneacetic acid) was developed as a brain penetrant structural analog of GABA (reviewed by Bryans and Wustrow, 1999). Gabapentin is approved for clinical use in the treatment of refractory partial seizures and secondary generalized tonic-clonic seizures but is being investigated as treatment for a number of disorders including bipolar disorder, social phobias, neuropathic pain, dental pain, osteoarthritis, and migraine. The mechanism(s) of gabapentin action is of high therapeutic importance, but remains unknown. Taylor et al. (1998) have published several comprehensive reviews of gabapentin pharmacology.

We were led to hypothesize that gabapentin could be an agonist at $GABA_B$ receptors based on its structural relatedness to baclofen (Bryans and Wustrow, 1999), and overlapping distribution of gabapentin binding with $GABA_B$ receptors in cortex, hippocampus, and cerebellum (Kaupmann et al., 1997, 1998a,b; Jones et al., 1998; Taylor et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999a,b). The hypothesis is also consistent with the mapping of the $GABA_B$ gb1 receptor gene to chromosome 6p21.3 in the vicinity of a susceptibility locus (EJM1) for idiopathic generalized epilepsy (Kaupmann et al., 1998a). We provide herein the first evidence that gabapentin is a selective agonist at the gb1a-gb2 heterodimer and postsynaptic $GABA_B$ receptor in situ that supports the existence of pharmacologically distinct $GABA_B$ receptor subtypes. This represents a potentially important breakthrough in the mechanism of action of the novel anticonvulsant drug gabapentin and suggests that postsynaptic $GABA_B$ gb1a-gb2 receptors coupled to inwardly rectifying potassium channels in limbic brain regions may be important drug targets for the treatment of epilepsy and other CNS disorders.

Materials and Methods

Receptor Expression Constructs. The open reading frame of human gb1a, gb1b, and gb1c isoforms were obtained from human cerebellum cDNA (CLONTECH, Palo Alto, CA) by polymerase chain reaction cloning using Advantage-HF polymerase chain reaction kit (CLONTECH) and primers based on gb1a (GenBank accession no. AJ225028) and gb1b (GenBank accession no. A225029) mRNA sequences deposited in GenBank. The cloning of the human gb2 receptor DNA (GenBank accession no. AF069755) has been reported elsewhere (Ng et al., 1999b). The native gb2 clone, or a gb2 construct encoding a modified influenza hemagglutinin signal sequence (MKTIIALSYIFCLVFA) followed by an antigenic FLAG (DYKDDDDK) epitope, or a gb2 construct encoding the bovine $GABA_A$ α1 signal sequence (MKKSPGLSDYLWAWTLFLSTLTGRSYGQPSLQD) followed by c-*myc* (EQKLISEEDLN) epitope were used for transient expression in *Xenopus laevis* oocytes. All $GABA_B$ receptor DNAs were subcloned into the pT7TS *X. laevis* oocyte expression vector (a gift from Dr. Paul Krieg). M2 muscarinic receptor and GsI cDNAs were generously supplied by BioSignal (Montréal, Canada).

$GABA_B$ Ligands. Gabapentin was extracted from Neurontin capsules (10 capsules, containing 400 mg of gabapentin) in boiling ethanol. After filtration through celite, the solid was triturated in isopropanol (30 ml) to give 3.21 g of a solid containing 85% gabapentin and 15% dextrose. Pure gabapentin was obtained by extraction of the celite cake in boiling methanol, filtration of the light suspension at room temperature, and trituration of the residue in ether to yield 1.00 g of a white solid. The white solid was further purified using preparative high-performance liquid chromatography with online mass spectrometric detection. The collected peak was evaporated to dryness and reconstituted for NMR analysis. The mass spectral and NMR data were consistent with gabapentin. Gabapentin was also obtained commercially (Sigma). Gabapentin was stored at −20°C, and freshly prepared and used immediately in the functional assays. GABA, the active enantiomer (*R*)-baclofen, and CGP55845 were purchased from Sigma and Tocris Cookson, respectively. CGP71872 was synthesized as reported previously (Belley et al., 1999).

***X. laevis* Oocyte Expression.** *X. laevis* oocytes were isolated and recordings were performed as described previously (Ng et al., 1999a). cDNA constructs for various Kir (Kir 3.1 or Kir 3.2) channel isoforms; human gb1a, gb1b, and gb1c; murine gb1a; human c-*myc*-gb2; FLAG-gb2 constructs; human M2 muscarinic receptor; human β_2-adrenergic receptor; and bovine Gsα were linearized by restriction enzymes and purified using Geneclean (Bio 101, Vista, CA). Capped mRNA was made using T7 RNA polymerase and the mMessage mMachine (Ambion, Austin, TX). Individual oocytes were injected with 5 to 10 ng (in 25–50 nl) of various murine or human gb receptors and human Kir 3.1/3.2 or with the β_2AR/Gsα or M2 muscarinic receptor coexpressed with Kir 3.2. Recordings were made at room temperature using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA). Oocytes were voltage clamped and perfused continuously with different recording solutions. Data was recorded at a holding potential of −80 mV and drugs were added to the bath with a fast perfusion system. Data collection and analysis were performed using pCLAMP v6.0 (Axon Instruments) and Origin v4.0 software (MicroCal, Northampton, MA).

Hippocampal Slices and Whole-Cell Recordings. Transverse hippocampal slices (300 μm) were obtained from male Sprague-Dawley rats (29–40 days postnatal) as described previously (Chapman and Lacaille, 1999). Individual slices were submerged in a chamber mounted on an upright microscope (Axioskop FS; Zeiss, Oberkochen, Germany) and perfused with ACSF at room temperature containing 124 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM dextrose, saturated with 95% O_2 and 5% CO_2, and at a flow rate of 2.5 to 3.0 ml/min. CA1 pyramidal neurons were visualized using differential-interference-contrast microscopy and an infrared CCD camera (Cohu 6500). Patch pipettes (4–8 MΩ) were filled with 140 mM K-gluconate, 5 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, 0.5 mM EGTA, 2 mM ATP-tris, 0.4 mM GTP-tris, 10 mM phosphocreatine, 0.1% biocytin, pH adjusted to 7.2 to 7.3 with KOH. Whole-cell, voltage-clamp re-

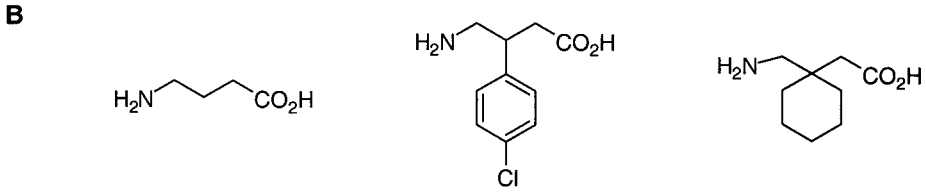
cordings were made with an Axopatch 200B amplifier (Axon Instruments) with low-pass filtering at 2 kHz. Currents were digitized and stored for future analysis (pClamp; Axon Instruments). Voltage measurements were corrected for liquid junction potentials (Neher, 1992). All drugs were bath applied. Baclofen and gabapentin currents were obtained, as described previously (Nurse and Lacaille, 1999), using membrane potential ramps and a subtraction procedure. I-V relations were obtained during membrane potential ramps from −60 to −160 mV over 800 ms, first in control ACSF and then in the presence of the drug. Averaged currents were obtained from six successive responses in each condition. Agonist currents were isolated by subtracting currents in control ACSF from currents in the presence of the agonist ($I_{agonist} = I_{I\text{-}V,\ agonist} - I_{I\text{-}V,\ acsf}$). Chord conductance measures were obtained at $V_m = -80$ mV for agonist currents using the formula $G_m = I/(V_m - E_{rev})$, where E_{rev} was the observed mean reversal potential for the agonist. The theoretical E_K was calculated using the formula $E_K = RT/F \times \ln [K]_o/[K]_i$. Monosynaptic fast $GABA_A$ inhibitory postsynaptic currents (IPSCs) were evoked with ultrasmall concentric bipolar electrodes (Frederick Haer) placed in stratum radiatum near the pyramidal neuron and using constant current pulses (15–90 μA, 0.5 ms) during blockade of non-NMDA and NMDA synaptic transmission with 20 μM CNQX (RBI) and 50 μM (±)-2-amino-5-phosphopentanoic acid (RBI, Natick, MA), respectively. Histological procedures for revealing biocytin-filled cells were as described previously (Chapman and Lacaille, 1999). Axonal and dendritic arborizations of filled cells were examined with a light microscope equipped with a CCD camera. Data are reported as mean ± S.E.M, unless otherwise noted.

Results

Molecular Characterization of gb1 Isoforms. With the aim of identifying pharmacologically distinct human $GABA_B$ receptors, we have cloned the open reading frame for human gb1a and gb1b isoforms and a new gb1c isoform (GenBank accession numbers AJ225028, A225029, and AJ012187, respectively) from adult human cerebellum mRNA. Gb1a, gb1b, and gb1c are proteins of 961, 844, and 899 amino acids, respectively. The three human gb1 isoforms differ only in the N-terminal sequence that precede a domain that is homologous to the bacterial periplasmic leucine-binding protein (Kaupmann et al., 1997; Galvez et al., 1999) (Fig. 1A). The gb1a-specific N-terminal sequence primarily comprises two protein-protein interacting Sushi Repeat (also known as short consensus repeat) domains of ~60 amino acids, the first corresponding to T^{26} to R^{98} and the second to K^{102} to N^{160}, first described by Kaupmann et al. (1998a) for this receptor. Gb1b differs from gb1a in that the first 164 amino acids of gb1a are replaced by 47 different amino acids; thus, gb1b lacks both N terminus Sushi Repeats. The novel gb1c isoform differs from gb1a by an in-frame 62 amino acid deletion and elimination of one Sushi Repeat, leaving a single Sushi Repeat interacting module. The human gb1c isoform is also structurally distinct from the rat $GABA_B$R1c, which exhibits an in-frame insertion of 31 amino acids between the second extracellular loop and the fifth transmembrane domain [Pfaff et al. (1999) and references therein). In light of recent studies that showed the N-terminal domain of gb1a to be sufficient to specify agonist and antagonist binding (Galvez et al., 1999; Malitschek et al., 1999), we asked whether the human gb1 isoforms, which differ in their ligand binding N-terminal domains, differ functionally in response to $GABA_B$ ligands, including gabapentin (Fig. 1B), when expressed in the absence or presence of gb2.

Gabapentin Agonism of $GABA_B$ gb1-gb2 Heterodimers Coupled to Kir Channels Expressed in *X. laevis* Oocytes. $GABA_B$ receptor-mediated K^+ membrane conductance was used as the functional assay to characterize the activity of gabapentin in this study, because unlike negative coupling of the receptor to adenylyl cyclase, for which the physiological role is unclear, a primary physiological role of $GABA_B$ receptors is to mediate increased K^+ membrane conductance in hippocampal neurons, leading to membrane

Fig. 1. A, amino acid alignment of the extracellular N-terminal domains of human gb1a, gb1b, and gb1c isoforms. The proposed signal peptide cleavage site of gb1 is marked with scissors. ▼, putative *N*-glycosylation sites; arrows delimit the Sushi domains (SU). →, start of the leucine-binding protein-like domain. Gaps (. . .) were introduced to maximize alignment. B, $GABA_B$ heterodimer pan agonists GABA and baclofen, and gb1a-gb2 heterodimer selective agonist gabapentin.



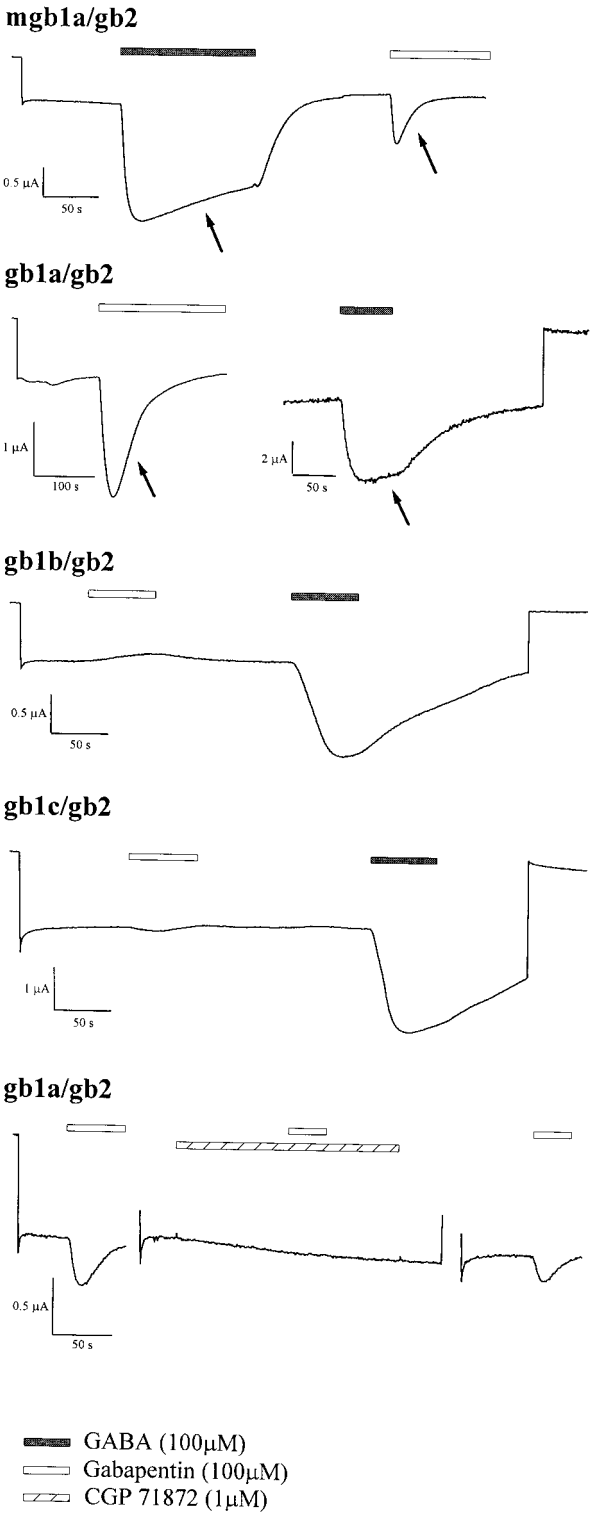


Fig. 2. Modulation of Kir 3.1/3.2 in *X. laevis* oocytes by different gb1-gb2 heteromers. Currents were measured by holding oocytes at −80 mV. The dark bar in each trace denotes changing from perfusion of oocytes with KD-98 solutions to solution containing 100 μM GABA. The light bar denotes the beginning of perfusion 100 μM gabapentin. Coexpression of human gb2 with mouse gb1a (first trace) human gb1a (second trace), gb1b (third trace), or gb1c (fourth trace). Block of 100 μM gabapentin response at gb1a-gb2 by 1 μM CGP 71872 followed by wash-out and reactivation by gabapentin is shown in trace five. Arrows denote different patterns of desensitization for GABA- or gabapentin-mediated receptor activation. Each trace is representative of at least four separate experiments performed on different oocytes.

hyperpolarization and inhibition of neuronal excitability (Sodickson and Bean, 1998; Bowery and Enna, 2000). We asked if gb1a and gb1b and the novel gb1c isoform expressed as homomers or heterodimers with gb2 could couple with Kir 3.1/3.2 channels in *X. laevis* oocytes, an established model for studying GPCR-activated inward rectifiers (Kir channels) (Dascal, 1997). Human or murine gb1a, human gb1b, and human gb1c isoforms were inactive when expressed individ-

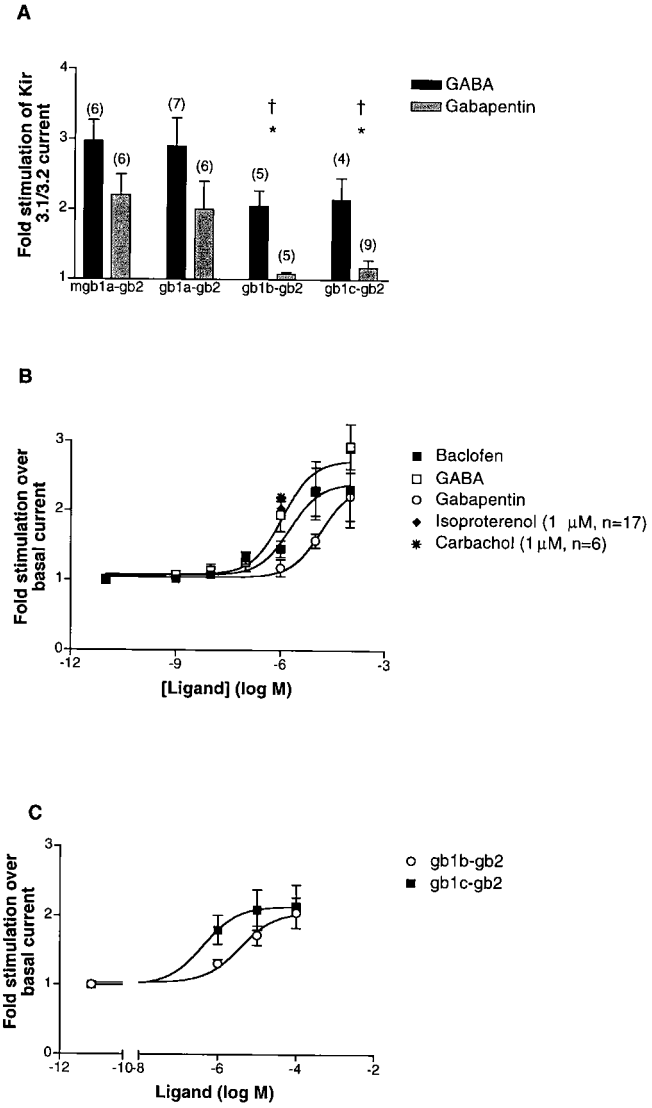


Fig. 3. Gabapentin modulates Kir 3.1/3.2 selectively via gb1a-gb2 receptor heteromers. A, fold stimulation of Kir 3.1/3.2 current by gabapentin or GABA over basal current (set to 1.0). No difference in GABA responses was detected among heterodimer subtypes ($p < 0.05$). Fold stimulation of GABA and gabapentin was similar at the gb1a-gb2 heterodimer ($p < 0.05$). Significant differences in gabapentin response among the subtypes (†) and between GABA versus gabapentin at the gb1b- and gb1c-gb2 heterodimers (∗) are indicated. Fold stimulation was calculated by dividing maximal ligand-stimulated current by basal current measured in KD-98 solution. Statistical analysis was determined by two tail Student's *t* test. B, dose-response relation for $GABA_B$ ligands at gb1a-gb2. Coexpression of murine gb1a and human gb2 modulates Kir 3.1/3.2 in a dose-dependent manner. Currents were measured at various doses of GABA, gabapentin, or baclofen and normalized relative to the basal current. C, dose-response relation for GABA at gb1b-gb2 and gb1c-gb2. Currents were measured at various doses of GABA and normalized relative to the basal current. Averaged data from many experiments (minimum of $n = 4$ for each point on the two dose-response curves) was pooled and fit to the Hill equation.

ually (data not shown). All require coexpression with human gb2 to form structurally distinct $GABA_B$ receptor heterodimer subtypes that can couple to Kir 3.1/3.2 channels. The three heterodimer subtypes are activated by GABA to a similar extent ($p > 0.05$): 2.9 ± 0.4-fold for gb1a-gb2 ($n = 6$), 2.05 ± 0.22-fold for gb1b-gb2 ($n = 5$), and 2.14 ± 0.31-fold for gb1c-gb2 ($n = 4$) (Figs. 2 and 3A). However, gabapentin (100 μM) only significantly activated Kir 3.1/3.2 channels through the gb1a-gb2 heterodimer (2.0 ± 0.4-fold stimulation, $n = 6$) compared with the other subtypes, $p < 0.05$ (Figs. 2 and 3A). Gabapentin agonism was indistinguishable at the mouse gb1a-gb2 heterodimer and could be blocked completely but reversibly by 1 or 0.1 μM CGP71872, a $GABA_B$ antagonist potent at nanomolar concentrations (Figs. 2 and 3A). In contrast to the results obtained with gb1a-gb2, only small and inconsistent responses to gabapentin were detected after stimulation of gb1b-gb2 (1.08 ± 0.02-fold stimulation, $n = 5$) or gb1c-gb2 (1.17 ± 0.11-fold stimulation, $n = 9$) even though GABA-mediated responses were always detected in the same oocytes and the extent of the GABA response was similar among the heterodimer subtypes (Figs. 2 and 3A). Furthermore, 100 μM gabapentin could not block the agonist effect of 10 μM GABA on gb1b/gb2 or gb1c/gb2 receptors (no block of GABA response for gb1b/gb2, $n = 3$; and 2 ± 2% block of gb1c/gb2, $n = 3$) arguing against the notion that gabapentin behaves as an antagonist or partial agonist at these subtypes. Collectively, our data suggest that gabapentin is a gb1a-gb2 heterodimer subtype-selective agonist.

Another difference between the responses to GABA and gabapentin was manifested by the rapid and consistent desensitization of the response to gabapentin during its continual presence (Fig. 2), essentially resulting in a return to basal current levels during 1 min of stimulation. A markedly reduced desensitization was occasionally detectable in responses to GABA (Fig. 2) or baclofen (data not shown). As for stimulation of $GABA_B$ receptors by GABA, only modest desensitization of current was detected during stimulation by M2- or βAR agonists (data not shown) consistent with reports in the literature using the oocyte expression system (Fidler-Lim et al., 1995; Doupnik et al., 1997).

The potency of GABA at gb1a- and gb1b-gb2 heterodimers was similar (1.1 versus 3.8 μM), consistent with previous pharmacological studies [Bowery and Enna (2000) and references therein]. GABA potency was higher at the gb1c-gb2 heterodimer (0.4 μM; Fig. 3, B and C), although the extent of GABA stimulation was not statistically different among the heterodimer subtypes. Studies of gabapentin dose dependence at gb1a-gb2 heterodimers revealed an EC_{50} value of 15 μM (Fig. 3B). Gabapentin agonism could be blocked by 10 nM or 1 uM CGP71872, and was reversible as reactivation by gabapentin was observed following washout of the antagonist (Fig. 2). Given the relative micromolar affinity of gabapentin (EC_{50} ~15 μM) and nanomolar affinity of CGP71872 (K_i ~0.5 nM; Belley et al., 1999) the ability of 10 nM CGP71872 to confer antagonism is not unexpected. The potency of gabapentin at the gb1a-gb2 heterodimer is in good agreement with its therapeutic dose, corresponding to 10 to 100 uM in brain, as monotherapy in the treatment of epilepsy or neuropathy (Backonja et al., 1998; Rowbotham et al., 1998; Bryans and Wustrow, 1999). This suggests that one possible mechanism by which gabapentin exerts its therapeutic actions is by selective $GABA_B$ gb1a-gb2 subtype agonism.

Gabapentin Actions in Rat Hippocampal Neurons In Situ. We next examined whether gabapentin was active at native $GABA_B$ receptors in CA1 pyramidal neurons of rat hippocampal slices (Luscher et al., 1997) (Fig. 4). Membrane currents evoked by bath application of gabapentin were isolated using voltage ramps and a subtraction procedure during whole-cell, patch-clamp recordings (Nurse and Lacaille, 1999). Currents obtained from the I-V relation in control ACSF were subtracted from those in the presence of gabapentin (Fig. 4B). Bath application of 1 mM gabapentin activated outward currents at membrane potentials near rest (Fig. 4B). These gabapentin currents reversed and became inward near −100 mV (mean $E_{rev} = -101.0 \pm 2.2$ mV, $n = 7$ cells). Because the calculated equilibrium potential for K^+ in our conditions was similar (−101.3 mV), the gabapentin-activated currents in CA1 pyramidal neurons were thus mediated by K^+. Gabapentin currents were dose-dependent, their mean chord conductance increasing with doses between 0.01 and 1 mM (Fig. 4, B and D). Bath application of 2 to 20 μM baclofen elicited similar potassium currents that were outward at membrane potentials near rest, reversed near −100 mV (mean $E_{rev} = -101.5 \pm 2.6$ mV, $n = 6$ cells) and were dose-dependent (Fig. 4, C and D). Potassium currents activated by 1 mM gabapentin and 20 μM baclofen were coupled to $GABA_B$ receptors since they were antagonized by pretreatment with the $GABA_B$ receptor antagonist CGP55845 (4 and 1 μM, respectively, Fig. 4D). The antagonism of gabapentin currents by CGP55845 was dose-dependent. Concentrations of 2 and 4 μM CGP55845 reduced currents evoked by 0.5 mM gabapentin by 86 and 100% respectively, and those elicited by 1 mM gabapentin by 61 and 89%, respectively (Fig. 4E). Thus, gabapentin activated potassium currents linked to postsynaptic $GABA_B$ receptors in CA1 pyramidal cells in situ, and these effects were similar to the postsynaptic actions of baclofen.

Because neuronal $GABA_B$ receptors are also located presynaptically and are coupled to inhibition of transmitter release (Misgeld et al., 1995; Kerr and Ong, 1995; Wu and Saggau, 1997; Bowery and Enna, 2000), we assessed whether gabapentin also activated presynaptic $GABA_B$ receptors that inhibit GABA release in hippocampal neurons in situ (Davies et al., 1990). Fast monosynaptic GABA IPSCs were evoked in CA1 pyramidal cells by electrical stimulation of stratum radiatum in the presence of blockers of non-NMDA and NMDA glutamate synaptic transmission (CNQX and AP5, respectively) (Fig. 5A) (Davies et al., 1990). These fast outward IPSCs recorded near resting membrane potential were mediated by $GABA_A$ receptors, because they were completely antagonized by 25 μM bicuculline ($n = 11$ cells, data not shown). Fast $GABA_A$ IPSCs were reversibly reduced by 72% by bath application of 20 μM baclofen (Fig. 5B). This presynaptic inhibition by baclofen was dose dependent (2–20 μM) and was antagonized by the $GABA_B$ receptor antagonist CGP55845 (1 μM; Fig. 5D). In contrast, gabapentin (0.01–1 mM) did not significantly depress $GABA_A$ IPSCs (Fig. 5, C and D), even during bath applications that elicited outward currents in the same cells (not shown). The small and variable reduction in IPSC amplitude observed in the presence of gabapentin (6–13%) was not significantly different from the reduction observed during repeated application of control ACSF (7%) or after pretreatment with the $GABA_B$ antagonist CGP55845 (16%) (Fig. 5D). This minor reduction, therefore, does not result from gabapentin effects. Our observations indicate that al-

though baclofen activated presynaptic $GABA_B$ receptors and inhibited GABA release in rat hippocampus, gabapentin did not. Thus, gabapentin does not have presynaptic actions similar to those of baclofen in CA1 hippocampus in situ.

Discussion

The three human gb1 N-terminal variants are inactive as homomers and require coexpression with gb2 to form functional and structurally distinct $GABA_B$ gb1a-gb2, gb1b-gb2, and gb1c-gb2 receptor heterodimer subtypes. This suggests that the novel human gb1c isoform like gb1a and gb1b forms a functional $GABA_B$ receptor with gb2 in the brain. Although GABA could stimulate all three subtypes of $GABA_B$ heterodimers coupled to Kir 3.1/3.2 channels in *X. laevis* oocytes, gabapentin (up to 100 μM) is an efficient agonist only at the gb1a-gb2 heterodimer subtype with negligible activity at gb1b-gb2 and gb1c-gb2 heterodimers. The murine gb1a receptor exhibits 98.5% amino acid identity to human gb1a (Ng et al., 1999a) and, as a heterodimer with gb2, could also

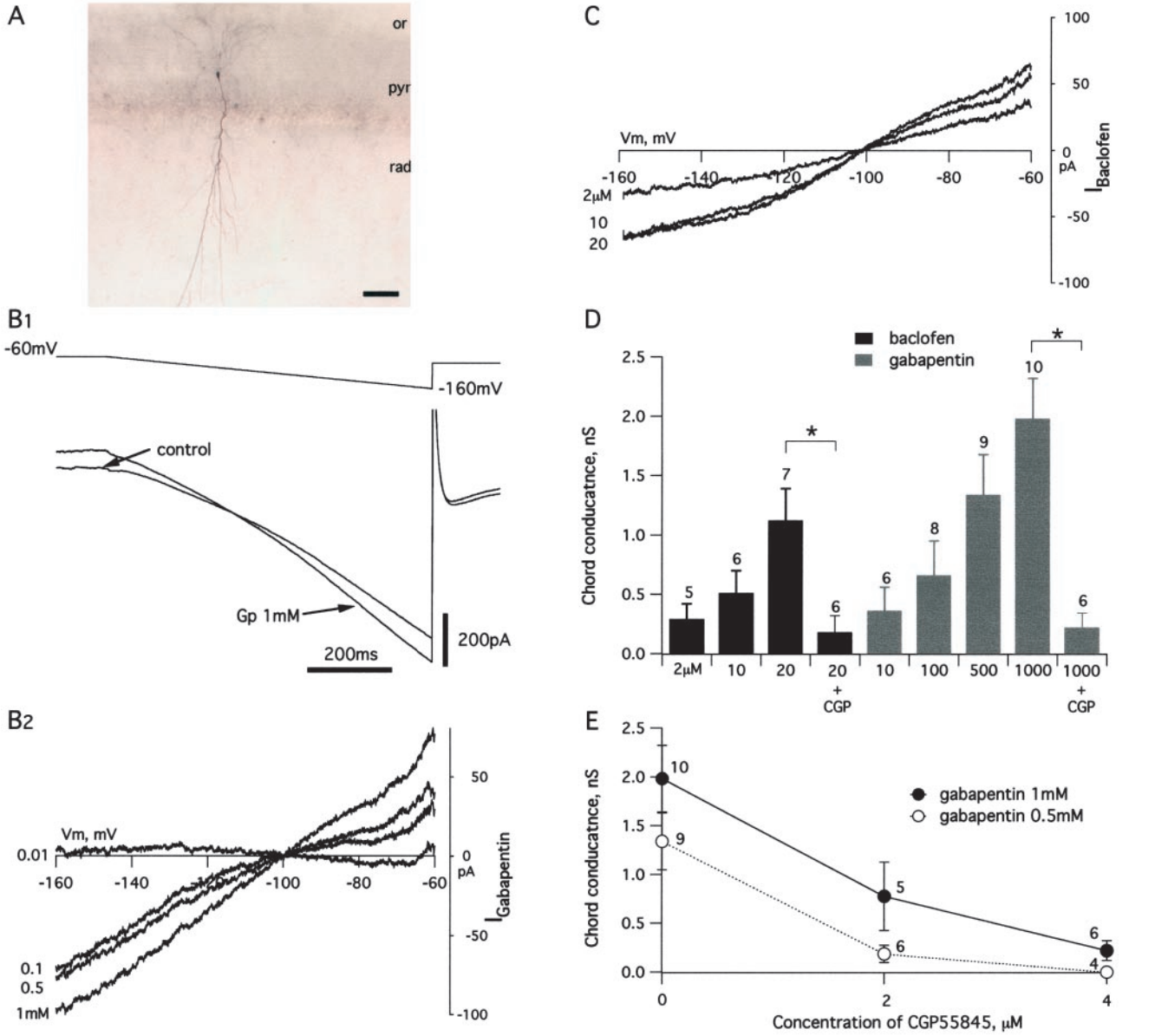


Fig. 4. Gabapentin activates potassium currents via $GABA_B$ receptors in CA1 pyramidal cells in situ. A, micrograph of a biocytin filled CA1 pyramidal neuron exposed to Gabapentin. Calibration mark 100 μm. B, I-V relations were obtained during voltage ramps from −60 to −160 mV in control ACSF and in the presence of 1 mM gabapentin (Gp; B_1). Gabapentin currents (B_2) were isolated by subtracting currents from I-V relations in control ACSF from those in the presence of gabapentin. The current evoked by 1 mM gabapentin in B_2 was obtained from the traces shown in B_1. Gabapentin evoked outward currents at membrane potentials between −60 and −100 mV. In the same cell, gabapentin currents increased in magnitude with increasing doses (0.1–1 mM). C, bath application of 2 to 20 μM baclofen elicited in CA1 pyramidal cells similar potassium currents with a comparable reversal potential. D, the mean chord conductance (measured at −80 mV) of baclofen and Gabapentin currents increased in dose-dependent fashion (the number above each bar indicates the number of cells tested). The dose-response relationship for gabapentin was shifted approximately 10-fold higher relative to that of baclofen. The mean chord conductance of gabapentin (1 mM) and baclofen (20 μM) potassium currents were blocked by the $GABA_B$ antagonist CGP55845 (at 4 and 1 μM concentration, respectively). E, the mean chord conductance of gabapentin currents (0.5 and 1 mM) was blocked in a dose-dependent manner by the $GABA_B$ antagonist CGP55845 (2 and 4 μM).

mediate gabapentin agonism with similar maximal stimulation confirming that gabapentin is an agonist at the gb1a-gb2 heterodimer subtype. Our data also show that gabapentin could not prevent GABA-mediated activation of gb1b/gb2 or gb1c/gb2 heterodimers, suggesting that it is not an antagonist at these receptors. The inability of gabapentin to block GABA responses and the inconsistent and weak stimulation of channel activity suggest that gabapentin is not a partial agonist at gb1b/gb2 receptors. Studies of dose dependence showed that gabapentin exhibits an EC_{50} value of ~15 μM at gb1a-gb2, but was inactive up to 100 μM in functional assays at the recombinant $GABA_A$ $\alpha1\beta3\gamma2$, $\alpha3\beta3\gamma2$, and $\alpha4\beta3\gamma2$ receptor subtypes (data not shown). Taken together, this is the first functional evidence that gabapentin is a selective $GABA_B$ receptor agonist. Studies performed using native tissues have suggested the existence of pharmacologically distinct pre- and postsynaptic receptor subtypes (reviewed by Bowery and Enna, 2000); definitive evidence, however, has been lacking, primarily because of the lack of selective $GABA_B$ receptor ligands. We show that gabapentin is a selective agonist at the gb1a-gb2 heterodimer subtype coupled to Kir channels and is the first selective pharmacological tool for this receptor system.

A structural basis for this pharmacological difference among heterodimer subtypes is probably attributable to the 62 amino acids specific to the N terminus of gb1a that contain a Sushi Repeat (K^{102}-N^{160}) that is absent in the gb1b and gb1c subunits. The extracellular N-terminal domain of $GABA_B$ receptors is postulated to form two lobes that upon agonist binding results in the entrapment of the ligand via a Venus Flytrap mechanism similar to that reported for bacterial periplasmic amino acid binding proteins (Galvez et al., 1999). Our data suggests that the 62 N-terminal amino acids specific to gb1a, with or without the participation of gb2, contain important determinants for gabapentin binding and entrapment. Consistent with this notion, the entire extracellular N terminus of gb1a is needed to retain the pharmacological characteristics of the full-length receptor (Malitschek et al., 1999, Sullivan et al., 2000). Gabapentin agonism can be competitively inhibited by CGP55845 antagonist (Fig. 5D), but as reported previously, agonist and antagonist binding sites in gb1a are specified by different N-terminal amino acid residues (Galvez et al., 1999). Site-directed mutagenesis studies will be needed to identify the determinants for gabapentin binding, because its micromolar potency for the gb1a-gb2 heterodimer excludes, as with agonists at the metabotropic glutamate receptors, detailed characterization by radioligand binding experiments.

A novel finding of our study is that the response to gabapentin desensitizes rapidly, unlike responses to GABA or

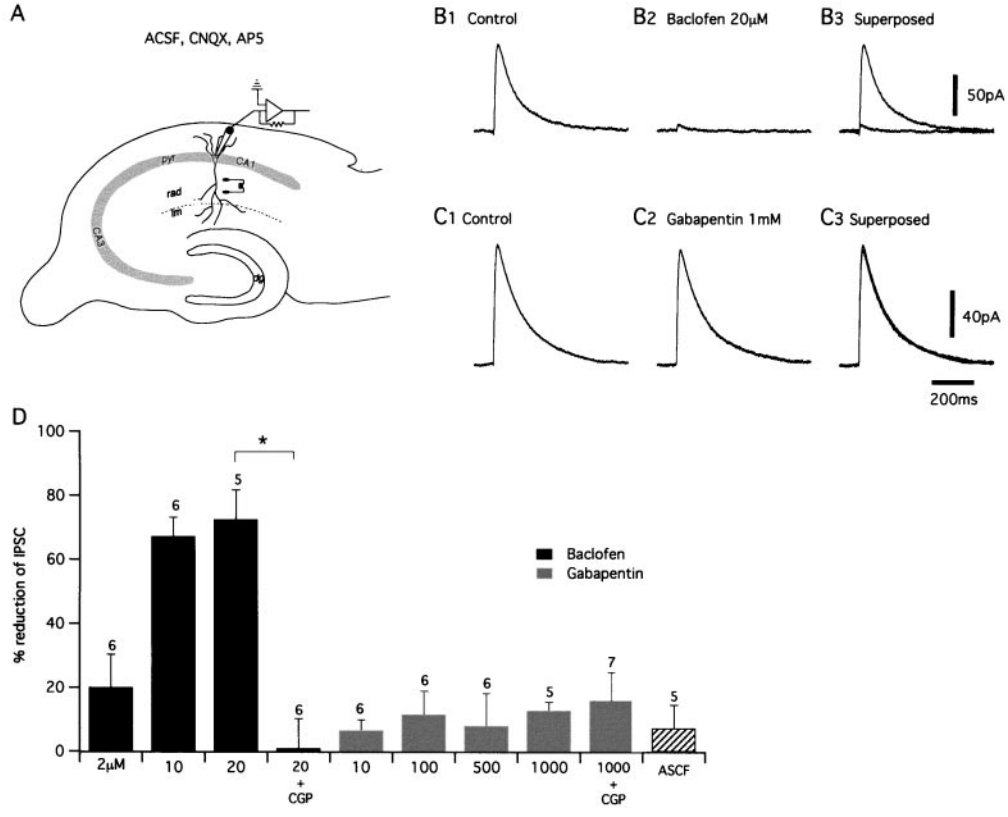


Fig. 5. Presynaptic $GABA_B$ inhibition of GABA synaptic transmission in hippocampus: inefficacy of gabapentin and efficacy of baclofen. A, experimental arrangement for evoking monosynaptic fast $GABA_A$ IPSCs by electrical stimulation of inhibitory fibers in stratum radiatum in the presence of blockers of glutamate synaptic transmission (20 μM CNQX and 50 μM AP5) during whole-cell, voltage-clamp recording from pyramidal cells. B, at resting membrane potential, stimulation evoked fast outward IPSCs in control ACSF (with CNQX and AP5). IPSCs were depressed during bath application of 20 μM baclofen. C, in contrast, during bath application of 1 mM Gabapentin, IPSCs were minimally affected relative to control. D, the mean reduction of IPSCs by baclofen was dose dependent (2–20 μM) and the presynaptic actions of 20 μM baclofen were completely antagonized by 1 μM the $GABA_B$ antagonist CGP55845. Gabapentin at concentrations between 0.01–1 mM did not produce such reductions in IPSC amplitude. The numbers above the bars indicate the number of cells tested in each condition. The small mean reduction in the presence of gabapentin was not significantly different from that seen with repeated application of control ACSF (with CNQX and AP5; diagonal bars) or during application of 4 μM CGP55845.

baclofen. This is unlikely to involve modulation by protein kinases as in classical GPCR desensitization (Ferguson et al., 1998), because the onset of this "desensitized" state is much more rapid by comparison. This acute desensitization has been detected in other studies of Kir channel modulation by G protein-coupled receptors (Chuang et al., 1998). It has been demonstrated that this desensitization can be accounted for by alterations in the hydrolysis cycle of the G proteins, which are the intermediates between the receptor and channel (Chuang et al., 1998). In native tissues, rapid onset of desensitization of GPCR-mediated Kir activation is a normal physiological response (Doupnik et al., 1997; Sodickson and Bean, 1998), which can be rescued in heterologous expression systems by coexpression with RGS proteins. RGS proteins can augment the rate of GTP hydrolysis and therefore nucleotide turnover (Doupnik et al., 1997) leading to more rapid activation and deactivation of GPCR-mediated responses. The reason for the differences in the rates of desensitization of responses to GABA and gapapentin are unclear but may involve a greater degree of GTP turnover stimulated by the latter.

GABA inhibition in the CNS involves multiple mechanisms. These include fast postsynaptic inhibition via activation of $GABA_A$ receptor chloride channels; slow postsynaptic inhibition via activation of $GABA_B$ receptors; G protein-regulated, inwardly rectifying potassium channels; and presynaptic inhibition via negative modulation of Ca^{2+} channels in presynaptic terminals reducing glutamate and GABA release (Nicoll et al., 1990; and reviewed by Kerr and Ong, 1995; Misgeld et al. 1995; Bowery and Enna, 2000). Fast postsynaptic $GABA_A$ responses result from activity at single synapses, whereas slower $GABA_B$ responses necessitate the synchronous activation of multiple presynaptic fibers (Otis and Mody, 1992). In addition, postsynaptic $GABA_B$ receptors seem important for curtailing epileptiform activity in the presence of impaired $GABA_A$ inhibition (Malouf et al., 1990; Scanziani et al., 1991). In hippocampal neurons, postsynaptic $GABA_B$ receptor activation leads to membrane hyperpolarization, mediated by inwardly rectifying potassium channels (Luscher et al., 1997). Furthermore, subcellular localization studies show that gb1a is predominantly postsynaptic, whereas gb1b is largely presynaptic (Benke et al., 1999; Fritschy et al., 1999), although one study reports a different conclusion (Billinton et al., 1999). The present data show for the first time that gabapentin is a selective agonist for the $GABA_B$ gb1a-gb2 heterodimer subtype coupled to Kirs and activated potassium currents linked to postsynaptic $GABA_B$ receptors in CA1 pyramidal cells in situ, providing the first in situ evidence of structurally and pharmacologically distinct pre- and postsynaptic $GABA_B$ receptor subtypes.

The lack of pharmacological agents selective for $GABA_B$ receptor subtypes has hampered the therapeutic use of $GABA_B$-related compounds. Indeed, because of such complex pre- and postsynaptic $GABA_B$ inhibitory mechanisms, nonselective $GABA_B$ compounds can have drastic opposite effects on paroxysmal depolarizing responses in CNS neurons: $GABA_B$ receptor activation can be proconvulsant via presynaptic mechanisms (Mott et al., 1989) and anticonvulsant via postsynaptic mechanisms (Malouf et al., 1990; Scanziani et al., 1991). Thus, the selective agonism of postsynaptic $GABA_B$ receptors in hippocampal neurons by gabapentin may be its therapeutic advantage as an anticonvulsant, because it would not act preferentially at presynaptic $GABA_B$ sites to reduce GABA release and thus would not induce the disinhibition that other nonselective agonists at presynaptic $GABA_B$ receptors provoke.

Acknowledgments

We thank Dr. Ken Koblan (Merck Research Laboratories, West Point, PA) for the NMDA NR2B receptor results, and Dr. Warren Y.K. Ng (Columbia University and New York Presbyterian Hospital, Columbia Presbyterian Center, New York, NY) for discussions of gabapentin clinical use.

References

Backonja M, Beydoun A, Edwards KR, Schwartz SL, Fonseca V, Hes M, LaMoreaux L and Garofalo E (1998) Gabapentin for the symptomatic treatment of painful neuropathy in patients with diabetes mellitus: A randomized controlled trial. *JAMA* **280:**1831–1836.

Belley M, Sullivan R, Reeves A, Evans J, O'Neill GP and Ng GYK (1999) Synthesis of the nanomolar photoaffinity $GABA_B$ receptor ligand CGP71872 reveals diversity in the tissue distribution of $GABA_B$ receptor forms. *Bioorg Med Chem* **7:**2697–2704.

Benke D, Honer M, Michel C, Bettler B and Mohler H (1999) γ-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 and Bowery NG (1999) $GABA_B$ receptor isoforms GBR1a and GBR1b, appear to be associated with pre- and post-synpatic elements respectively in rat and human cerebellum. *Br J Pharmacol* **126:**1387–1392.

Bowery NG and Enna SJ (2000) γ-Aminobutyric $acid_B$ receptors First of the functional metabotropic heterodimers. *J Pharmacol Exp Ther* **292:**2–7.

Bryans JS and Wustrow DJ (1999) 3-Substituted GABA analogs with central nervous system activity: A review. *Med Res Rev* **19:**149–177.

Chapman CA and Lacaille J-C (1999) Cholinergic induction of theta-frequency oscillations in hippocampal inhibitory interneurons and pacing of pyramidal cell firing. *J Neurosci* **19:**8637–8645.

Chuang H-H, Yu M, Jan Y-N, and Jan LY (1998) Evidence that the nucleotide exchange and hydrolysis cycle of G proteins causes acute desensitization of G protein-gated inward rectifier K^+ channels. *Proc Natl Acad Sci USA* **95:**11727–11732.

Dascal N (1997) Signalling via the G protein-activated K+ channels. *Cell Signal* **9:**551–573.

Davies CH, Davies SN and Collingridge GL (1990) Paired-pulse depression of monosynaptic GABA-mediated inhibitory postsynaptic responses in rat hippocampus. *J Physiol (Lond)* **424:**513–531.

Doupnik CA, Davidson N, Lester HA and Kofuji P (1997) RGS proteins reconstitute the rapid gating kinetics of Gβγ-activated inwardly rectifying K^+ channels. *Proc Natl Acad Sci USA* **94:**10461–10466.

Doze VA, Cohen GA and Madison DV (1995) Calcium channels involvement in $GABA_B$ receptor-mediated inhibition of GABA release in area CA1 of the rat hippocampus. *J Neurophysiol* **74:**43–53.

Ferguson SSG, Zhang J, Barak LS and Caron MG (1998) Molecular mechanisms of G protein-coupled receptor desensitization and resensitization. *Life Sci* **62:**1561–1565.

Fidler-Lim N, Dascal N, Labarca C, Davidson N and Lester HA (1995) A G protein-gated K channel is activated via β2-adrenergic receptors and Gβγ subunits in *Xenopus* oocytes. *J Gen Physiol* **105:**421–439.

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

Galvez T, Parmentier M-L, Joly C, Malitschek B, Kaupmann K, Kuhn R, Bittiger H, Froestl W, Bettler B and Pin J-P (1999) Mutagenesis and modeling of the $GABA_B$ receptor extracellular domain support a venus flytrap mechanism for ligand binding. *J Biol Chem* **274:**13362–13369.

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

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

Kaupmann K, Schuler V, Mosbacher J, Bischoff S, Bittiger H, Heid J, Froestl W, Leonhard S, Pfaff T, Karchin A and Bettler B (1998a) Human γ-aminobutyric acid type B receptors are differentially expressed and regulate inwardly rectifying K+ channels. *Proc Natl Acad Sci USA* **95:**14991–14996.

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

Kerr DIB and Ong J (1995) $GABA_B$ receptors [review]. *Pharmacol Ther* **67:**187–246.

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

Luscher C, Jan LY, Stoffel M, Malenka RC and Nicoll RA (1997) G protein-coupled inwardly rectifying K+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. *Neuron* **19:**687–695.
Malitschek B, Schweizer C, Keir M, Heid J, Froestl W, Mosbacher J, Kuhn R, Henley J, Joly C, Pin J-P, Kaupmann K and Bettler B (1999) The N-terminal domain of γ-aminobutyric acid (B) receptors is sufficient to specify agonist and antagonist binding. *Mol Pharmacol* **56:**448–454.
Malouf AT, Robbins CA and Schwartzkroin PA (1990) Phaclofen inhibition of the slow inhibitory postsynaptic potential in hippocampal slice cultures: A possible role for the $GABA_B$-mediated inhibitory postsynaptic potential. *Neuroscience* **35:** 53–61.
Martin SC, Russek SJ and Farb DH (1999) Molecular identification of the human $GABA_BR2$: Cell surface expression and coupling to adenylyl cyclase in the absence of $GABA_BR1$. *Mol Cell Neurosci* **13:**180–191.
Misgeld U, Bijak M and Jarolimek W (1995) A physiological role for $GABA_B$ receptors and the effects of baclofen in the mammalian central nervous system. *Prog Neurobiol* **46:**423–462.
Mott DD, Bragdon AC, Lewis DV and Wilson WA (1989) Baclofen has a proepileptic effect in the rat dentate gyrus. *J Pharmacol Exp Ther* **249:**721–725.
Neher E (1992) Correction for liquid junction potentials in patch clamp experiments. *Methods Enzymol* **207:**123–131.
Ng GYK, Clark J, Coulombe N, Ethier N, Hebert T, Sullivan R, Kargman S, Chateauneuf A, Tsukamoto N, MacDonald T, Whiting P, Mezey E, Johnson MP, Liu JQ, Kolakowski FL Jr, Evans J, Bonner T and O'Neill GP (1999a) Identification of a $GABA_B$ receptor subunit, gb2, required for functional $GABA_B$ receptor activity. *J Biol Chem* **274:**7607–7611.
Ng GYK, MacDonald T, Bonnert T, Rigby M, Heavens R, Whiting P, Chateauneuf A, Coulombe N, Kargman S, Caskey T, Evans J, O'Neill GP and Liu Q (1999b) Cloning of a novel G protein-coupled receptor GPR51 resembling $GABA_B$ receptors expressed predominantly in nervous tissues and mapped proximal to the hereditary sensory neuropathy type 1 locus on chromosome 9. *Genomics* **56:**288–295.
Nicoll RA, Malenka RM and Kauer JA (1990) Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. *Physiol Rev* **70:**513–565.
Nurse S and Lacaille J-C (1999) Late maturation of $GABA_B$ synaptic transmission in area CA1 of the rat hippocampus. *Neuropharmacology* **38:**1733–1742.
Otis TS and Mody I (1992) Differential activation of $GABA_A$ and $GABA_B$ receptors by spontaneously released transmitter. *J Neurophysiol* **67:**227–235.
Pfaff T, Malitschek B, Kaupmann K, Prezeau L, Pin J-P, Bettler B and Karschin A (1999) Alternative splicing generates a novel isoform of the rat metabotropic $GABA_BR1$ receptor. *Eur J Neurosci* **11:**2874–2882.
Rowbotham M, Harden N, Stacey B, Bernstein P and Magnus-Miller L (1998) Gabapentin for the treatment of postherpetic neuralgia: A randomized controlled trial. *JAMA* **280:**1837–1842.
Scanziani M, Gähwiler BH and Thompson SM (1991) Paroxysmal inhibitory potentials mediated by $GABA_B$ receptors in partially disinhibited rat hippocampal slice cultures. *J Physiol (Lond)* **444:**375–396.
Sodickson DL and Bean BP (1998) Neurotransmitter activation of inwardly rectifying potassium currents in dissociated hippocampal CA3 neurons: Interactions among multiple receptors. *J Neurosci* **18:**8153–8162.
Sullivan R, Chateauneuf A, Coulombe N, Kolakowski LF Jr, Johnson MP, Hebert TE, Ethier N, Belley M, Metters M, Abramovitz M, O'Neill GP and Ng GYK (2000) Coexpression of full-length γ-aminobutyric $acid_B$ ($GABA_B$) receptors with truncated receptors and metabotropic glutamate receptor 4 supports the $GABA_B$ heterodimer as the functional receptor. *J Pharmacol Exp Ther* **293:**460–467.
Taylor CP, Gee NS, Su T-Z, Kocsis JD, Welty DF, Brown JP, Dooley DJ, Boden P and Singh L (1998) A summary of mechanistic hypotheses of gabapentin pharmacology. *Epilepsy Res* **29:**233–249.
White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM and Marshall FH (1998) Heterodimerization is required for the formation of a functional $GABA_B$ receptor. *Nature (Lond)* **396:**679–682.
Wu L-G and Saggau P (1997) Presynaptic inhibition of elicited neurotransmitter release. *Trends Neurosci* **20:**204–212.

Send reprint requests to: Dr. Gordon Y. K. Ng, Merck Frosst Center for Therapeutic Research, 16711 Trans Canada Hwy., Kirkland, Quebec, Canada (E-mail: gordon_ng@merck.com).