Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 14991–14996, December 1998
Neurobiology

Human γ-aminobutyric acid type B receptors are differentially expressed and regulate inwardly rectifying K^+ channels

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Communicated by Stephen F. Heinemann, The Salk Institute for Biological Studies, La Jolla, CA, October 6, 1998 (received for review June 15, 1998)

ABSTRACT **γ-Aminobutyric acid type B receptors ($GABA_BRs$) are involved in the fine tuning of inhibitory synaptic transmission. Presynaptic $GABA_BRs$ inhibit neurotransmitter release by down-regulating high-voltage activated Ca^{2+} channels, whereas postsynaptic $GABA_BRs$ decrease neuronal excitability by activating a prominent inwardly rectifying K^+ (Kir) conductance that underlies the late inhibitory postsynaptic potentials. Here we report the cloning and functional characterization of two human $GABA_BRs$, $hGABA_BR1a$ (hR1a) and $hGABA_BR1b$ (hR1b). These receptors closely match the pharmacological properties and molecular weights of the most abundant native $GABA_BRs$. We show that in transfected mammalian cells hR1a and hR1b can modulate heteromeric Kir3.1/3.2 and Kir3.1/3.4 channels. Heterologous expression therefore supports the notion that Kir3 channels are the postsynaptic effectors of $GABA_BRs$. Our data further demonstrate that in principle either of the cloned receptors could mediate inhibitory postsynaptic potentials. We find that in the cerebellum hR1a and hR1b transcripts are largely confined to granule and Purkinje cells, respectively. This finding supports a selective association of hR1b, and not hR1a, with postsynaptic Kir3 channels. The mapping of the *$GABA_BR1$* gene to human chromosome 6p21.3, in the vicinity of a susceptibility locus (*EJM1*) for idiopathic generalized epilepsies, identifies a candidate gene for inherited forms of epilepsy.**

GABA (γ-aminobutyric acid), the most abundant inhibitory neurotransmitter in the mammalian central nervous system, activates ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. $GABA_A$ receptors convey fast synaptic inhibition by activating a Cl^- conductance that is allosterically modulated by many psychoactive drugs, such as the benzodiazepines, barbiturates, and neurosteroids. $GABA_BRs$ are coupled to G proteins and have been implicated in synaptic inhibition (1), hippocampal long-term potentiation (2), slow wave sleep (3), muscle relaxation, and antinociception (4). Many of the physiological roles of $GABA_BRs$ can be attributed to the modulation of G protein-gated Ca^{2+} and K^+ channels. It has been suggested that pharmacologically distinct $GABA_BR$ subtypes mediate pre- and postsynaptic actions (see ref. 5 and refs. therein).

$GABA_BRs$, first recognized 18 years ago by Bowery *et al.* (6), are abundant in the central nervous system. Although $GABA_BRs$ have been described early on, they remained the last of the major neurotransmitter receptors to be cloned (7). This was due to the difficulties in coupling $GABA_BRs$ to effector channels in heterologous cells, which prevented expression cloning strategies such as those commonly used for the isolation of neurotransmitter receptors. The first $GABA_BR$ cDNA was eventually isolated by using a radioligand-binding screening approach (7). Two N-terminal splice variants, $rGABA_BR1a$ (rR1a) and $rGABA_BR1b$ (rR1b), have been characterized in rats. The rR1a and rR1b receptors share intracellular effector domains and ligand-binding sites, they are expressed throughout the brain, and their transcript distribution qualitatively parallels that of radioligand-binding sites (7–9). The rank order of drug-binding affinities at native and recombinant receptors is identical, indicating that rR1a and rR1b can account for the majority of native $GABA_BR$ sites. In contrast to native $GABA_BRs$, the cloned receptors have a 100-fold reduced agonist affinity, which could for instance indicate inefficient coupling to G proteins in transfected cells. Present knowledge does not rule out the possibility that all actions of native receptors are related to R1a and R1b (5). Accordingly, molecular studies have not yet substantiated the claim for additional $GABA_BRs$. In a first effort to reproduce native signaling by using the cloned receptors, we have demonstrated a negative coupling to adenylyl cyclase (7). The weak inhibition of forskolin-stimulated cAMP production prevented more detailed functional studies. A matching of cloned receptors with possible effector channels in heterologous expression systems would provide a more sensitive assay system. Several recent reports suggest that Kir3 inwardly rectifying K^+ channels (formerly GIRK) are prominent effectors of native $GABA_BRs$ (10–13). For example, $GABA_BR$-mediated K^+-dependent inhibition is impaired in the hippocampus of Kir3.2-deficient mice (10).

Herein, we describe the molecular structure and pharmacological and functional properties of human $GABA_BR1$. The binding pharmacology of the cloned hR1a and hR1b parallels that of the rat receptors. Notably the affinity for agonists is low when compared with native $GABA_BRs$. The heterologous coupling of $GABA_BR1$ to Kir3 channels allows for the first time a comparison of functional and binding data at cloned receptors. β-(4-Chlorophenyl)-γ-aminobutyric acid (baclofen), a $GABA_BR$ agonist, activates Kir3 channels with a potency that is similar to its binding affinity at recombinant receptors. This similarity may indicate that inefficient G protein coupling is not the cause of the low agonist-binding affinity at cloned receptors. Furthermore, based on functional and *in situ* hybridization data, we propose that R1b mediates postsynaptic inhibition in the cerebellum. Preliminary results of this study have been published in abstract form (9, 14).

Abbreviations: $GABA_BR$, $GABA_B$ receptor; hR1, rR1 human, rat $GABA_BR1$ (comprising R1a and R1b); R1a, $GABA_BR1a$; R1b, $GABA_BR1b$; baclofen, β-(4-chlorophenyl)-γ-aminobutyric acid; GABA, γ-aminobutyric acid; GPCR, G protein-coupled receptor; Kir, inwardly rectifying K^+ channel.

Data deposition: The hR1a and hR1b cDNA sequences reported in this paper have been deposited in the European Molecular Biology Laboratory (EMBL) database [accession nos. AJ225028 (hR1a) and AJ225029 (hR1b)].

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METHODS

Ligands. $GABA_BR$ selective ligands were synthesized in-house. [^{125}I]CGP64213 and [^{125}I]CGP71872 were labeled to a specific radioactivity of >2,000 Ci/mmol, [^{3}H]CGP54626A to 40–60 Ci/mmol (ANAWA AG, Wangen, Switzerland).

Cloning of hR1a and hR1b cDNAs. A human fetal brain cDNA library (CLONTECH) was screened by colony hybridization using a ^{32}P-labeled 1.3-kb *Pvu*II–*Sca*I fragment of rR1a cDNA as a probe (7). Membranes were hybridized overnight in 0.5 M NaH_2PO_4 (pH 7.2), 7% SDS, and 1 mM EDTA at 60°C and washed for 1 h with 2× SSC and 0.1% SDS at 50°C. Two overlapping clones comprised most of the coding region. The missing N-terminal sequences were cloned by using a 5′-RACE kit (Boehringer Mannheim). First strand cDNA was synthesized from human cerebellum poly(A)$^+$ RNA by using an hR1-specific primer (5′-TAGGGTTGTGGAGTGTGG-3′). After tailing, the cDNA was PCR amplified by using an oligo(dT) primer and a nested hR1-specific primer (5′-CTGGATCACACTTGCTGT-3′). PCR products were cloned into Bluescript SK(−) (Stratagene), and inserts encoding N-terminal sequences of hR1a and hR1b were sequenced. The cDNAs encoding the entire ORF were inserted into pC1-neo (Promega). Additionally a cDNA (4.4 kb) containing the entire ORF of hR1a was isolated from a human cerebellum cDNA library that was generated as described (7). The sequence of this clone is identical to that isolated by rapid amplification of cDNA ends-PCR.

Cell Culture, Ligand Binding Assays and Photoaffinity Labeling. Mammalian cells were purchased from the American Type Culture Collection (ATCC). HEK293, CHO, and CCL39 cells were transfected by using lipofection kits (Qiagen, Hilden, Germany), COS-1 cells were transfected by using a modified DEAE-Dextran procedure (7). Binding experiments, generation of stable cell lines, and [^{125}I]CGP71872 photoaffinity labeling were carried out as described (7).

Electrophysiology. Concatemers of Kir3.1/3.2 and Kir3.1/3.4 subunits (15) were subcloned into pSVSport1 (Life Technologies, Gaithersburg, MD). $GABA_BR1$ and Kir3 cDNAs were cotransfected at a 2:1 (wt/wt) ratio into semi-confluent HEK-293 cells or COS-7 cells (16). Whole-cell patch-clamp recordings were performed at room temperature 48–72 h after transfection in a bath solution consisting of 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM Hepes, pH 7.4. Patch pipettes were pulled from borosilicate glass capillaries, heat-polished to give input resistances of 3–5 MΩ, and filled with 140 mM KCl, 2 mM $MgCl_2$, 1 mM ethylene-bis(oxyethylenenitrilo) tetraacetate (EGTA), 1 mM Na_2ATP, 100 μM cAMP, 100 μM GTP, and 5 mM Hepes (pH 7.3). Currents were recorded with an EPC9 patch clamp amplifier, low pass-filtered at 1–2 kHz, and stored by using PULSE/PULSEFIT software (Heka, Lamprecht, Germany). Data analysis was performed with IGOR software (WaveMetrics, Lake Oswego, OR).

***In Situ* Hybridization, Northern Blot Analysis, and Autoradiographic Detection of $GABA_BR$-Binding Sites.** Cryosections of human (10 μm, male, 50 years of age, and no clinical or neuropathological signs) and rat cerebellum (10 μM) were used for *in situ* hybridization with [^{35}S]UTP or [^{35}S]ATP-labeled riboprobes encoding sequences common (pan) and unique to R1a and R1b. The hybridization probes for human and rat were: 466 bp *Pst*I–*Bgl*II/768 bp *Pst*I–*Sca*I (pan), 334 bp *Sma*I/406 bp *Sma*I (R1a), and 280 bp KspI/301 bp KspI (R1b), respectively. Post-hybridization was performed under high stringency conditions (human: 50% formamide, rat: 63% formamide, 80°C for the post-hybridization wash). Slides were dipped into nuclear emulsion and exposed for 15–45 days. Northern blots (CLONTECH) were hybridized at 50°C in 0.5 M NaH_2PO_4 (pH 7.2), 7% SDS, 1 mM EDTA, and 50% (vol/vol) formamide. Filters were washed with 0.1× SSC and 0.1% SDS at 68°C. The distribution of $GABA_BR$-binding sites was examined by using the selective antagonist [^{3}H]CGP54626 and autoradiography. Tissue sections were incubated for 90 min with 2 nM radioligand in the presence or absence of 10 μM baclofen and exposed for 10 days to Ultrofilm-^{3}H (Leica, Glattbrugg, Switzerland).

Chromosomal Localization. Genomic clones comprising mouse and human $GABA_BR1$ genes were obtained after filter hybridization of bacterial artificial chromosome (BAC) and phage artificial chromosome (PAC) libraries (Genome Systems, St. Louis) with ^{32}P-labeled cDNA probes. Genomic DNA was labeled with digoxigenin UTP and hybridized (50% formamide, 10% dextran sulfate, and 2× SSC) to metaphase chromosomes derived from mouse embryonic fibroblasts and human peripheral blood lymphocytes. Hybridization was visualized by using a fluoresceinated anti-digoxigenin antibody.

RESULTS

Structure and Binding Pharmacology of Human $GABA_BRs$. The hR1a and hR1b are composed of 961 and 844 amino acids, respectively (Fig. 1*A*). Human and rat receptors are highly related; the mature proteins share 99% sequence identity (differences are underlined in Fig. 1*A*). A structural model, which emphasizes functional domains and sequence motifs present in $GABA_BRs$, is shown in Fig. 1*B*. The molecular weights of the cloned receptors expressed in mammalian cells closely match the molecular weights of native human $GABA_BRs$ (Fig. 1*C*). In line with this, high levels of hR1a (≈4.4 kb) and hR1b (≈4.3 kb) transcripts are expressed in brain (Figs. 1*D* and 3). Low levels of hR1a mRNA also are found in human heart tissue (Fig. 1*D*).

The pharmacological properties of the cloned receptors expressed in mammalian cells have been analyzed in competition binding experiments with [^{125}I]CGP64213 and $GABA_BR$-selective ligands (Table 1). The IC_{50}s for all agonists and antagonists tested match closely with the values for the rat receptors (7). This emphasizes the high degree of sequence conservation in the putative ligand-binding domain where human and rat receptors only differ in two positions (I403T and D413E, Fig. 1*A*). As for the rat receptors, no significant pharmacological difference is observed between hR1a and hR1b.

The low agonist-binding affinity of the rat receptors is reproduced with hR1a and hR1b (Table 1 and ref. 7). Inefficient G protein coupling or differences in the relative expression levels of receptor and the G proteins could explain low-affinity binding of agonists (17). However, we have determined similar affinities for GABA (EC_{50} values ranging from 30 to 50 μM) by using four cell clones that express high and low levels of $GABA_BR1a$ protein (the maximal numbers of [^{125}I]CGP64213-binding sites (B_{max}) were 3.6, 4.1, 22.3, and 43.7 pmol mg^{-1} protein, respectively). Moreover, agonist affinities are similar in COS-1, HEK 293, CHO-K1, and CCL-39 cells expressing $GABA_BR1$ (data not shown).

Coupling of hR1a and hR1b to Kir3 Channels. We have coexpressed hR1a and hR1b with concatenated pairs of both Kir3.1/3.2 or Kir3.1/3.4 subunits (Fig. 2). The Kir3 currents mediated by concatemeric subunits are indistinguishable from the typical inwardly rectifying currents evoked by separate pairs of subunits (15). A functional coupling of hR1a and hR1b to Kir3 channels is evident from the macroscopic properties of GABA- and baclofen-induced whole-cell currents (Fig. 2*A*). At a holding potential of −100 mV and $[K^+]_o$ of 25 mM, HEK 293 cells expressing hR1b and Kir3 subunits generate a basal inward current of −354 ± 180 pA (Kir3.1/3.4) and −279 ± 122 (Kir3.1/3.2). This current is enhanced ≈2-fold by application of 50 μM baclofen (−756 ± 428 pA for Kir3.1/3.4, 534 ± 369 for Kir3.1/3.2, n = 15). In our experiments, only 10% of the cells expressing Kir3 currents >200 pA (25 mM $[K^+]_o$) showed a positive coupling to $GABA_BR1$ receptors. For other G protein-coupled receptors (GPCRs) the yield of Kir3 coupling is significantly higher (50–75%, refs. 16 and 18). A $GABA_BR1$-green fluorescent protein (GFP) construct is expressed at levels comparable with other GPCRs (16, 18). Usually >80% of the cells analyzed showed I_{Kir} currents. Cells that exhibit I_{Kir} currents are

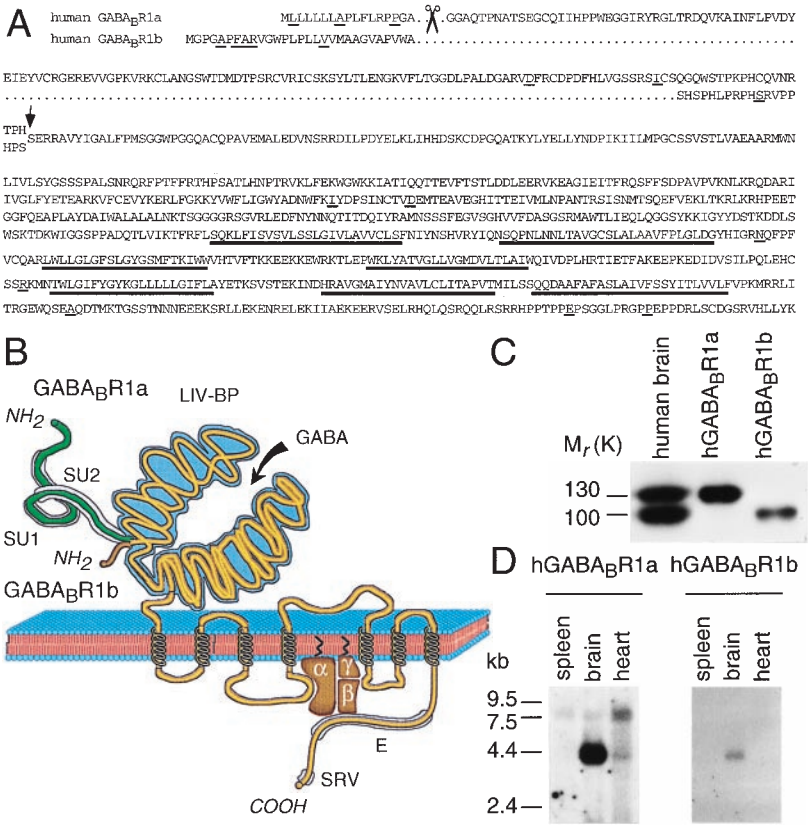

FIG. 1. Structure of hGABA$_B$Rs. (*A*) Predicted protein sequence of hR1a and hR1b. The arrow indicates the boundary between unique N-terminal hR1a and hR1b sequences, colored in *B* green and brown, respectively, and the common sequence. Amino acid residues that differ between rat and human receptors are underlined. Proposed signal peptide cleavage sites are marked with scissors. Putative transmembrane domains are underlined in bold. (*B*) Domain structure of GABA$_B$Rs. GABA$_B$Rs share extended sequence similarity with mGluRs, the Ca^{2+}-sensing receptor and a family of vomeronasal receptors (see ref. 5 and refs. therein). The mature hR1b protein differs from hR1a in that the N-terminal 147 residues are replaced by 18 different residues. The R1a-specific region mostly consists of two Sushi Repeats (SU1, SU2), ≈60 amino acid residues each, which occur in a variety of complement and adhesion proteins that engage into protein–protein interactions (42). The N-terminal domain that is common to R1a and R1b shares similarity to a guanylate cyclase intracellular domain and to bacterial periplasmic amino acid-binding proteins, such as the leucine–isoleucine–valine-binding protein (LIV-BP). Based on the three-dimensional structure of a LIV-BP, one expects this domain to fold into two lobes that constitute the GABA-binding site. The C-terminal domain is enriched in glutamic acid residues (E). At position −6 from the C terminus, GABA$_B$Rs display a putative PDZ-interacting module (serine/arginine/valine, SRV) (see ref. 5 and refs. therein), which may direct the assembly of signal transduction complexes. Specificity for G protein coupling is likely provided by the second intracellular loop, as for the mGluRs. (*C*) [^{125}I]CGP71872 photoaffinity labeling of membranes from rat cortex and COS-1 cells transfected with hR1a and hR1b cDNAs. Autoradiography of a SDS/PAGE (6%) is shown. (*D*) Northern blot analysis of hR1a and hR1b expression. Blots with 2 μg human poly(A)$^+$ RNA per lane were hybridized to selective ^{32}P-labeled probes.

expected to express GABA$_B$R1 because the corresponding cDNA has been transfected in excess. Therefore, a low rate of Kir3-coupled GABA$_B$R1 likely reflects inefficient assembly of the signaling cascade. Voltage-step and ramp protocols of both basal and baclofen-induced currents reveal a current-voltage (I–V) relationship with strong rectification (Fig. 2*A*). Baclofen-induced I_{Kir} reveal amplitudes of similar magnitude independent of whether hR1a or hR1b were expressed in COS-7 or HEK 293 cells (data not shown). No current desensitization after repetitive hR1b stimulation was detectable (Fig. 2*B*). The onset of current activation is rapid and follows a single exponential time course (τ_{ON} = 189 ± 97 ms, n = 12; Fig. 2*C*). Current relaxation occurs with similarly fast kinetics (τ_{OFF} = 223 ± 81 ms, n = 12). The rapid current activation suggests a membrane delimited regulation of Kir3 channels by the βγ subunits (Gβγ) of the G protein (12, 19, 20).

The half-maximally EC$_{50}$ of baclofen at hR1a (11.3 μM, Fig. 2 *D* and *E*) and GABA$_B$Rs expressed in cerebellar granule cells (K_d = 16 μM, ref. 21) is similar. These values also are similar to the binding affinity of baclofen at recombinant receptors (≈30 μM, Table 1). This may indicate that the cause of the low affinity of agonists at recombinant GABA$_B$R1, when compared with native GABA$_B$Rs, is not necessarily inefficient G protein coupling. Other factors, e.g., posttranslational modifications or associated proteins, may therefore regulate agonist affinity at GABA$_B$Rs. Alternatively, it cannot be excluded that only a minor fraction of recombinant GABA$_B$R1 exhibit high agonist affinity and those are the receptors that couple to Kir3 channels. The antagonist CGP54626A inhibits baclofen-induced currents with a potency (Fig. 2*F*) that is similar to its binding affinity (Table 1). Similar results have been obtained by using the cloned rat receptors (data not shown). A recent report shows that baclofen elicits I_{Kir} in *Xenopus* oocytes coinjected with cerebellar poly(A)$^+$ RNA and Kir3.1/3.2 cRNAs (22). We have expressed GABA$_B$R1 together with Kir3.1/3.2 in *Xenopus* oocytes and did not detect any GABA$_B$R-mediated current modulation. Additional factor(s), likely supplemented with the cerebellar poly(A)$^+$ RNA, seem to be necessary for a functional coupling in the oocyte.

Spatial Distribution of GABA$_B$R1 Splice Variants. The R1a/R1b mRNA distribution in human and rat brain was studied by *in situ* hybridization (Fig. 3). The two receptor variants are abundantly expressed in all major brain structures (7). Qualitatively, the distribution of transcripts in rat and human tissue sections appears similar. In the cerebellum R1a and R1b transcripts are sequestered to distinct cellular compartments. R1a transcripts are mostly confined to the granular cell and molecular

Table 1. Ligand-binding affinities at cloned human $GABA_B$ receptors

Ligand	$GABA_BR1a$		$GABA_BR1b$	
	IC_{50}, μM	Hill coeff. n_H	IC_{50}, μM	Hill coeff. n_H
Agonists				
GABA	30.5 ± 3.4	0.82 ± 0.02	30.9 ± 2.9	1.03 ± 0.08
APPA	2.5 ± 0.6	0.82 ± 0.07	2.5 ± 0.4	0.87 ± 0.02
Baclofen	31.8 ± 2.3	0.85 ± 0.06	30.1 ± 4.0	0.89 ± 0.14
CGP47656	14.8 ± 0.9	0.73 ± 0.03	13.9 ± 1.4	0.69 ± 0.12
Antagonists				
CGP56999A	0.0007 ± 0.0001	1.33 ± 0.12	0.0006 ± 0.0002	1.30 ± 0.27
CGP62349	0.0011 ± 0.0002	0.85 ± 0.09	0.0007 ± 0.0001	0.80 ± 0.13
CGP64213	0.0023 ± 0.0003	1.00 ± 0.09	0.0019 ± 0.0004	0.97 ± 0.06
CGP54626A	0.0019 ± 0.0004	1.06 ± 0.06	0.0014 ± 0.0004	0.96 ± 0.13
CGP71872	0.0030 ± 0.0003	1.00 ± 0.12	0.0026 ± 0.0006	0.93 ± 0.07
CGP35348	20.1 ± 2.5	0.82 ± 0.09	15.8 ± 3.3	0.72 ± 0.03
2-OH-saclofen	67.9 ± 4.4	0.90 ± 0.11	49.1 ± 11.2	0.76 ± 0.04
Saclofen	310 ± 57	0.69 ± 0.16	299 ± 59	0.53 ± 0.02
SCH50911	0.35 ± 0.02	0.86 ± 0.15	0.35 ± 0.05	0.93 ± 0.04

Inhibition of [^{125}I]CGP64213 binding to recombinant human $GABA_BRs$ by $GABA_B$ agonists and antagonists. Membranes from transiently transfected COS-1 cells (hR1a) and stably transfected CHO-K1 (hR1b) were used. IC_{50} values and Hill coefficients were fitted by using nonlinear regression (PRISM 2.0, Graph Pad Software, Sand Diego). Values are means ± SEM of three independent experiments. APPA, 3-aminopropylphosphinic acid.

layer whereas R1b transcripts are abundant in Purkinje cells. Pan probes that detect both the R1a and R1b transcripts allow to compare the relative level of expression in cerebellar layers. The highest levels of mRNA expression is found in Purkinje cells, moderate levels of transcripts are present in the granular cell layer, and low levels are found in the molecular layer. In general, less hybridization signal is obtained with human tissue.

Consistent with the [^{3}H]CGP54626-binding data (Fig. 3) $GABA_BR$-binding sites are reported to be present in the molecular layer and to a much lower extent in the granular cell layer of the cerebellum (8). In the absence of conclusive immunohistochemical evidence the expression of R1b mRNA in Purkinje cells supports an association of R1b protein with Purkinje cell dendrites, which process into the molecular layer

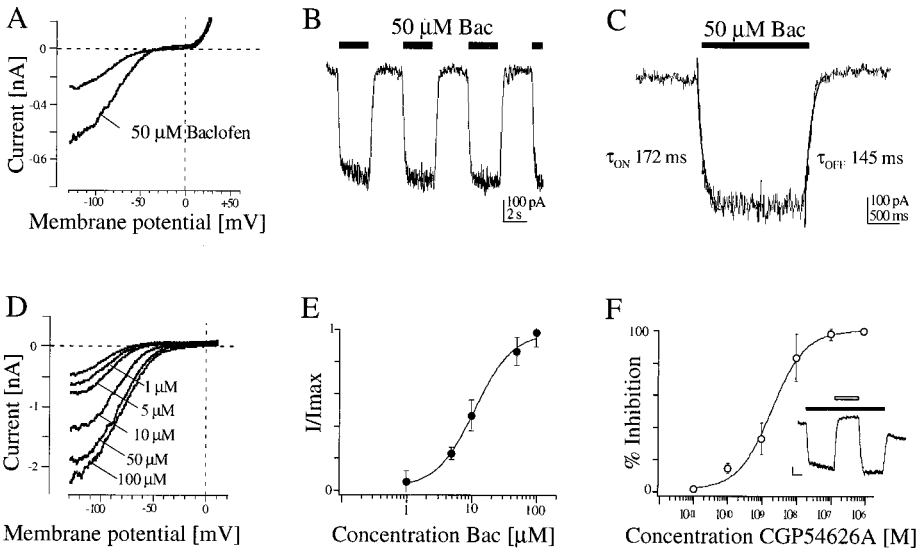

FIG. 2. The hR1a and hR1b activate heteromeric K^+ channels composed of Kir3.1/3.2 and 3.1/3.4 subunits. (*A*) Whole-cell currents recorded from HEK 293 cells transfected with cDNAs for hR1a receptors and Kir3.1/3.2 subunit concatemers in the absence and presence of 50 μM baclofen. Currents are responses to 1-s voltage ramps between −130 and +60 mV and show a selective increase of I_{Kir} currents by baclofen. (*B*) Current responses mediated by hR1b receptors to 50 μM baclofen at a holding potential (V_h) of −100 mV are nondesensitizing and do not decline upon repetitive application of agonist. (*C*) When applied through a microcapillary perfusion system that allowed solution exchanges within < 20 ms, the baclofen-induced currents activate and relax (upon removal of the agonist) with rapid time course, suggesting a direct G$\beta\gamma$-mediated channel activation. Onset and relaxation from activation are fitted to single exponentials with time constants τ_{ON} and τ_{OFF} as indicated. (*D* and *E*) Concentration/response relationships of recombinant $GABA_BR1$ for the agonist baclofen. Voltage ramp responses in *D* are basal and receptor-activated currents through Kir3.1/3.4 channels. For the plot in *E*, baclofen-induced current amplitudes at −80 mV are normalized to the maximal current amplitude obtained with 500 μM. Values are mean ± SD (n = 6) and are collected from both hR1a and hR1b. The least-squares fits are derived iteratively from $I = I_{max}\,[1/1+(EC_{50}/[A])^n]$ with I_{max} as the maximal current amplitude, EC_{50} as the half-maximal agonist concentration (11.3 μM), [A] as the agonist concentration, and n as the Hill coefficient (1.5). (*F*) Concentration/response relationships of hR1b for the specific $GABA_BR$ antagonist CGP54626A (n = 4). The percentage of inhibition of baclofen-induced currents (50 μM) is shown. Values are normalized to the inhibition obtained with 10 μM CGP54626A. An example of complete inhibition of baclofen-induced currents (black bar) by 100 nM CGP54626A (open bar) is shown in the insert (space bars denote 200 pA, 1 s). The least-squares fit reveal an IC_{50} of 2.0 nM and a Hill coefficient of 0.98 for CGP54626A. All recordings were done in 25 mM $[K^+]_o$.

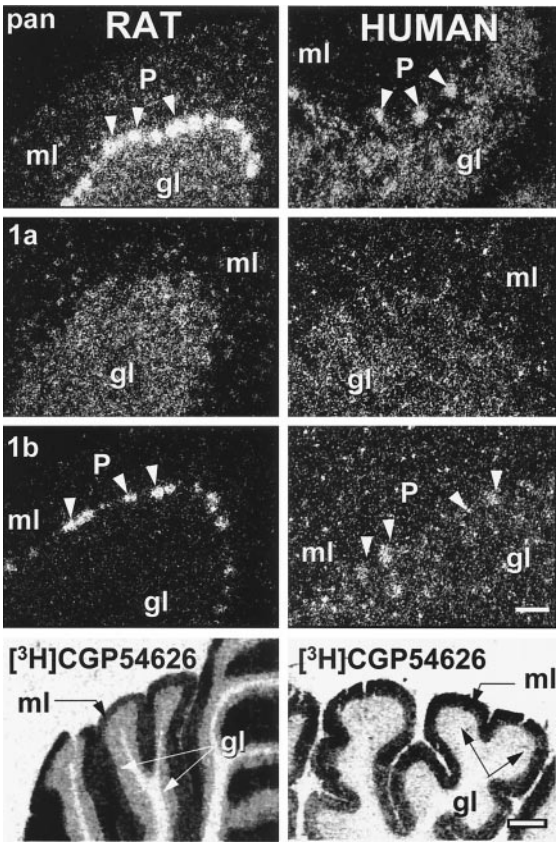

FIG. 3. Distribution of $GABA_BR$ transcripts and binding sites in rat and human cerebellum. Top panels show *in situ* hybridization data with probes that detect transcripts of R1a (1a), R1b (1b) or both (pan). The two Bottom panels show autoradiograms of $GABA_BR$-binding sites labeled with [^{3}H]CGP54626. Binding site density is high in the molecular layer as compared with the granular cell layer. Arrowheads indicate Purkinje cells. P, Purkinje cells; ml, molecular layer; gl, granular layer. [Scale bars: 0.1 mm (*in situ* hybridization), 0.4 mm (receptor autoradiography). Microscope images were computer-digitized under darkfield illumination at ×100 magnification. Slides were counterstained with cresyl violet to allow the identification of cell types under brightfield illumination.]

and are postsynaptic to stellate and basket cells. Likewise, the R1a protein is expected on the parallel fibers, which are excitatory to Purkinje cell dendrites in the molecular layer.

Chromosomal Localization of the *$GABA_BR1$* Gene. Alterations of $GABA_BR$ expression have been reported in animal models of absence epilepsy (23). To investigate a possible involvement of the cloned $GABA_BRs$ in inherited diseases, we have determined the chromosomal localization of *$GABA_BR1$* gene by fluorescence *in situ* hybridization (Fig. 4). The *$GABA_BR1$* gene maps on mouse chromosome 17B3 and human chromosome 6p21.3. The gene is localized close to the major histocompatibility complex (HLA). The localization of the human *$GABA_BR1$* gene on chromosome 6 is further supported by a transcript map of the HLA class I region (24). Two of the expressed sequence tags that have been characterized, GT545 and GT546, correspond to human *$GABA_BR1$*. These expressed sequence tags have been mapped in the HLA-F region close to the myelin/oligodendrocyte glycoprotein. *$GABA_BR1$* localizes to a region on chromosome 6p21.3 where a major susceptibility locus (*EJM1*) for common subtypes of idiopathic generalized epilepsy, comprising juvenile myoclonic epilepsy, juvenile absence epilepsy, and idiopathic generalized epilepsy with tonic clonic seizures on awakening, has been identified (25, 26).

DISCUSSION

$GABA_BR$ Modulation of Kir3 Channels. The hR1a/hR1b receptors activate Kir3.1/3.2 and Kir3.1/3.4 channels in transfected mammalian cells with an EC_{50} value for baclofen of 11.3 μM (Fig. 2*E*). $GABA_BRs$ of CA1 and CA3 hippocampal neurons (10 μM, ref. 27; 3 μM, ref. 12) and cerebellar granule cells (16 μM, ref. 21) activate K^+ channels with similar potency. Baclofen activates K^+ channels in deep dorsal horn neurons of the spinal cord with a higher potency (300 nM, ref. 28). This discrepancy may indicate $GABA_BR$ subtypes, multiple affinity states of the cloned receptor, or differences in the effector systems.

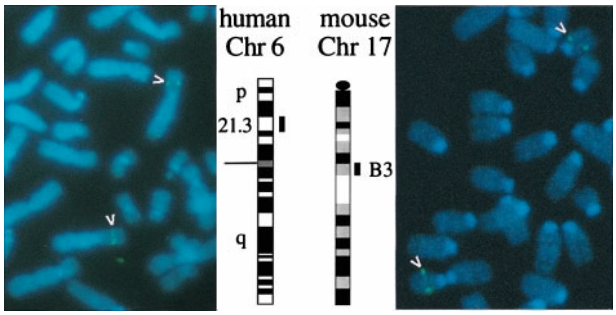

FIG. 4. Chromosomal localization of the *$GABA_BR1$* gene. Metaphase chromosomes were hybridized with digoxigenin-UTP-labeled probes derived from genomic PAC and BAC clones corresponding to the human and mouse *$GABA_BR1$* gene, respectively. Localization of the mouse gene to chromosome (Chr) 17 (arrowheads) was confirmed by cohybridization with a telomer-selective biotin-labeled probe. Hybridization of the human gene to the mid arm of chromosome (Chr) 6 (arrowheads) was confirmed by cohybridization with a centromer-selective biotin-labeled probe. A total of 80 metaphases for each species were analyzed.

The values for the time course of activation, the amplitude relation of basal and agonist-induced versus basal I_{Kir}, and the Hill coefficient of the $GABA_BR1$ to Kir3 channel coupling are similar to the values obtained with other GPCRs (16). However, the I_{Kir} does not desensitize in response to repetitive stimulation with baclofen. This may indicate that $GABA_BRs$, unlike many GPCRs, do not desensitize after phosphorylation/dephosphorylation in heterologous expression systems. The Hill coefficient of Kir3 activation by hR1a (1.5, Fig. 2*E*) is higher than in agonist-binding studies (0.85, Table 1) and is similar to values obtained for baclofen-activation of K^+ currents in cultured neurons (1.4–1.7, ref. 12). A likely step for cooperativity is the opening of Kir3 channels that requires the binding of more than one $G\beta\gamma$ complex (29, 30). Ruling out simple explanations, such as a low transfection efficiency, it remains unclear why a functional coupling is only obtained in a small subset of transfected cells. Additional factor(s) that direct receptors to their effectors may be limiting in the heterologous expression system. Candidates for such factors are the receptor–activity-modifying proteins (31), ODR-4 (32) or PDZ domain proteins (Fig. 1*B*, for refs. see 5).

Occasionally we have found that in transfected HEK 293 cells the basal I_{Kir} is inhibited after activation of $GABA_BR1$ (data not shown). This inhibition is fast and reversible and therefore unlike the slow and irreversible protein kinase C-mediated inhibition of I_{Kir} seen with other GPCRs (33, 34).

An important issue is whether $GABA_BR1$ coupling to Kir3 channels in heterologous expression systems reflects the situation in neurons. The Kir3.1/3.2/3.3 subunits are highly expressed in CA1 and CA3 pyramidal cells, in dentate gyrus granule cells, and in cerebellar granule cells (35). All these neuronal populations express $GABA_BR$ mRNA as well (7). The subunit composition of Kir3 channels in different hippocampal neurons has not been determined precisely. However, the single channel properties of recombinant Kir3.1/3.2 channels are reminiscent of those of K^+ channels activated by GPCRs in neurons (36). Furthermore, immunoprecipitation experiments have indicated that a majority of heteromeric Kir3 channels in the cerebral cortex, hippocampus, and cerebellum are assembled from Kir3.1 and Kir3.2 subunits (37). It has been recently demonstrated that in mice that

lack the Kir3.2 subunit the hyperpolarizing $GABA_BR$-activated K^+ current is absent. This suggests that the Kir3.1/3.2 are the main effector channels of postsynaptic $GABA_BRs$ (10, 21). Our experiments now show that the cloned $GABA_BR1$ receptors can reproduce a functional coupling to Kir3 in a heterologous expression system.

Seizures have been reported in Kir3.2 gene knock-out mice as well as in weaver (*wv*) mice, which carry a pore mutation in the Kir3.2 subunit (21, 38). This phenotype has been attributed to the impaired $GABA_BR$-mediated inhibition. It has been proposed that in the thalamus, $GABA_BR$-mediated inhibitory postsynaptic potentials have a priming function toward the generation of low threshold Ca^{2+} potentials, thereby facilitating burstfiring of the type observed in absence epilepsy (39). Animal models provide further evidence for a critical role of $GABA_BRs$ and associated effector systems (K^+/Ca^{2+} channels) in epilepsy. For example, $GABA_B$ antagonists suppress absence seizures whereas agonists exacerbate seizures in lethargic *(lh)* mice (23) and in a strain of rats (GAERS) with genetic absence epilepsy (40). This, together with the chromosomal localization, identify the $GABA_BR1$ gene as a valid candidate gene for inherited forms of idiopathic generalized epilepsy.

R1b Is a Candidate for Generating Late Inhibitory Postsynaptic Potentials. The high density of $GABA_BR$-binding sites in the cerebellar molecular layer, together with a distinct cellular distribution of transcripts, suggest that the R1a and R1b receptors target to distinct synaptic sites (Fig. 3). R1b is likely to be expressed on Purkinje cells dendrites that are postsynaptic to GABAergic stellate and baskets cells in the molecular layer. R1a transcripts are highly expressed in the granule cells, suggesting R1a protein in the parallel fiber terminals, which are excitatory to Purkinje cell dendrites in the molecular layer. Clearly, a definitive conclusion has to await ultrastructural studies with R1a- and R1b-selective antibodies. A study by using a nonselective antibody directed to the common C-terminal domain of R1a and R1b emphasizes that on GABAergic synapses in the rat retina, the cloned receptors are present at pre- and postsynaptic locations (41). It is tempting to speculate that R1b receptors are the native $GABA_BRs$ that mediate postsynaptic inhibition. The targeting of $GABA_BR1$ splice variants to distinct subcellular sites may therefore dictate effector preferences. This mechanism may generate functional diversity in the absence of a genetic diversity comparable to the mGluRs.

CONCLUSION

The major effectors of native $GABA_BRs$ are adenylyl cyclase, inwardly rectifying K^+ and high voltage-activated Ca^{2+} channels. The cloned receptors are now demonstrated to couple to Kir3 channels and to adenylyl cyclase in transfected cells, reinforcing that *in vivo*, many actions are likely to be mediated through these receptors. Considering that the cloned receptors are expressed presynaptically (41), we expect these receptors also to couple to Ca^{2+} channels. So far, all attempts to obtain a functional coupling to a Ca^{2+} conductance failed. Even now, after the cloning of $GABA_BRs$, the difficulties in demonstrating a robust coupling of cloned $GABA_BRs$ to ion channels in heterologous cells persist. This result suggests the involvement of additional factor(s) that are limiting or missing in nonneuronal expression systems. Associated proteins that direct GPCRs to the cell surface and alter their pharmacological properties, like the receptor–activity-modifying proteins (31) and ODR-4 (32), could be involved in the guiding of $GABA_BRs$ to effector channels. Such proteins also would provide an explanation for the differences in drug efficacies observed *in vivo*.

We thank D. Hoyer for providing human cerebellum sections, F. Döring for constructing the concatenated Kir3 channels, and E. Wischmeyer for stimulating the collaboration between Göttingen and Basel. This work was funded in part by the Deutsche Forschungsgemeinschaft (SFB406).

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