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Point Mutations in Either Subunit of the $GABA_B$ Receptor Confer Constitutive Activity to the Heterodimer[S]

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ABSTRACT

The GABA receptor ($GABA_BR$) is a class C G protein-coupled receptor (GPCR) that functions as an obligate heterodimer, composed of two heptahelical subunits, $GABA_BR$ subunit 1 (R1) and $GABA_BR$ subunit 2 (R2). In this study, we generated and pharmacologically characterized constitutively active $GABA_BR$ mutants as novel tools to explore the molecular mechanisms underlying receptor function. A single amino acid substitution, T290K, in the R1 agonist binding domain results in ligand-independent signaling when this mutant subunit is coexpressed with wild-type R2. Introduction of a Y690V mutation in the putative G protein-coupling domain of R2 is sufficient to confer moderate constitutive activity when this subunit is expressed alone. Activity of the Y690V mutant can be markedly enhanced with coexpression of wild-type R1. Coexpression of both mutant subunits (R1-T290K and R2-Y690K) leads to a further increase in basal signaling. Potencies of the full agonists *R*-(+)-β-(aminomethyl)-4-chlorobenzenepropanoic acid hydrochloride (baclofen) and GABA are increased at the constitutively active versus the corresponding wild-type receptors. The mutant $GABA_BR$ variants provided a sensitive probe enabling detection of inverse or partial agonist activity of molecules previously considered neutral antagonists. Our studies using constitutively active isoforms provide independent support for a model of $GABA_BR$ function that takes into account 1) ligand binding by R1, 2) signal transduction by R2, and 3) modulation of R2-induced function by R1. Furthermore, we demonstrate that certain hallmark features of constitutive activity as originally established with class A GPCRs (e.g., enhanced agonist potency and affinity), are more generally applicable, as suggested by our finding with a class C heterodimeric receptor.

GABA is an inhibitory neurotransmitter that acts through both ionotropic (type A) and metabotropic (type B) receptors. The $GABA_A$ receptor is a chloride channel that is rapidly gated in response to GABA binding. In contrast, the $GABA_B$ receptor ($GABA_BR$) belongs to the class C family of seven transmembrane domain G protein-coupled receptors (GPCRs), which also includes glutamate, calcium-sensing, pheromone, and sweet taste receptors. Most members of the class C family are known to form homodimers. In contrast, the $GABA_BR$ is an obligate heterodimer that is composed of two homologous subunits, $GABA_BR$ subunit 1 (R1) and $GABA_BR$ subunit 2 (R2) (Kaupmann et al., 1998; White et al., 1998). Each $GABA_BR$ subunit has a heptahelical domain as well as a large N-terminal region that includes a venus flytrap module (VFTM) (Margeta-Mitrovic et al., 2001). This N-terminal segment shares sequence similarity with certain bacterial periplasmic-binding proteins (O'Hara et al., 1993).

Unlike other class C receptors, the two subunits of the heterodimeric $GABA_BR$ play distinct roles in receptor activation. The VFTM of R1 confers agonist affinity (Takahashi et al., 1993; Pin et al., 2003), whereas R2 triggers G protein (G_i/G_o) activation (Galvez et al., 2001; Duthey et al., 2002). R2 also controls intracellular trafficking of R1, thereby enabling R1 to reach the cell surface (Margeta-Mitrovic et al., 2001; Pagano et al., 2001; Robbins et al., 2001). In addition to

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ABBREVIATIONS: $GABA_BR$, $GABA_B$ receptor; GPCR, G protein-coupled receptor; R1, $GABA_B$ receptor subunit 1; R2, $GABA_B$ receptor subunit 2; VFTM, venus flytrap module; PCR, polymerase chain reaction; HEK, human embryonic kidney; SRE, serum response element; CGP54626, [*S*-(*R**,*R**)]-[3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl)phosphinic acid; CGP52432, 3-[[(3,4-dichlorophenyl)methyl]amino]propyl] diethoxymethyl)phosphinic acid; CGP55845, (2*S*)-3-[[(1*S*)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid; CGP 35348, (3-aminopropyl)(diethoxymethyl)phosphinic acid; mGluR1, metabotropic glutamate receptor-subtype 1; CCK, cholecystokinin; CaSR, calcium sensing receptor; TM, transmembrane.

G_i/G_o-mediated inhibition of adenylate cyclase, $GABA_BR$ stimulation also leads to effector responses through G protein $\beta\gamma$ subunits. β-γ-mediated signaling includes activation of G protein-coupled inwardly rectifying potassium channels and inhibition of voltage-gated calcium channels.

The $GABA_BR$ is widely expressed in the peripheral and central nervous systems. Pharmacological modulation of this GPCR offers a potential treatment option for neurological disorders, including epilepsy, pain, anxiety, and spasticity (Vaught et al., 1985; Bowery et al., 2002; Sanger et al., 2002). *R*-(+)-β-(Aminomethyl)-4-chlorobenzenepropanoic acid hydrochloride (baclofen), a synthetic $GABA_BR$ agonist identified before the cloning of the receptor, has been used to treat spasticity for more than 30 years. Ongoing screening efforts in the pharmaceutical industry using the recombinant $GABA_BR$ are aimed at expanding the range of receptor selective drugs (Marshall, 2005).

Cumulative evidence suggests that agonist stimulation of a GPCR triggers a transition from the inactive to the active receptor state, leading to the induction of second messenger signaling (Gether, 2000). Even in the absence of agonist, however, many GPCRs exhibit a limited degree of ligand-independent (constitutive) signaling that is determined by the equilibrium between active and inactive conformations of the receptor (Adan and Kas, 2003). Constitutive activity of a given GPCR can be increased by introduction of receptor point mutations that further favor the active state. The majority of constitutively active receptors are "partially on" (i.e., stimulation with agonist leads to a further shift toward the active state and an increase in second messenger signaling).

Stimulation with agonist and induction of mutation-induced ligand-independent signaling offer complementary approaches to probe the mechanisms underlying receptor activation. Although activating point mutations have been described for many GPCRs, none has been reported for heterodimeric receptors, leading to the question of whether there are fundamental differences between homo- and heterodimeric receptors.

In the studies described here, we demonstrate that selected single amino acid substitutions in either $GABA_BR$ subunit result in significant constitutive activity of the heterodimers formed from an association of mutant and complementary wild-type subunits. Coexpression of both mutant subunits leads to an even more pronounced increase in basal signaling. In previous studies, it has been observed that ligand-independent signaling could be induced by either 1) introduction of a disulfide bridge within the putative ligand binding pocket or 2) coexpression of a wild-type and a chimeric R1/R2 subunit (Galvez et al., 2001; Kniazeff et al., 2004). In contrast to these $GABA_BR$ constructs, the constitutively active mutants described in our study show conserved ligand binding and agonist-stimulated function. These features enable further pharmacological analysis of the different constitutively active $GABA_BR$ mutants, thereby providing insight into the principles underlying signaling by a class C heterodimeric receptor.

Materials and Methods

Cloning of the Mouse R1 and R2 Subunits. PCR primers were designed to amplify the open reading frame of each mouse $GABA_BR$ subunit (Supplemental Table 1). As template for PCR, oligo(dT)-primed first-strand cDNA was generated by reverse transcription of mouse brain mRNA. For PCR amplification, the following parameters were used: 30 cycles, each including denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min. After the last cycle, a final extension period of 10 min at 72°C was completed.

Primers (Supplemental Table 1) were designed to amplify two overlapping segments of cDNA encoding the R1 subunit (GenBank accession no. AF008649). The two resulting PCR products were then ligated using a unique Bsu36I site and subcloned into the expression vector pcDNA1.1 (Invitrogen, Carlsbad, CA).

An analogous strategy was used to amplify the R2 cDNA. In this case, the 5′ primer (R2-A1 in Supplemental Table 1) was designed from the mouse R2 genomic sequence, which shares 100% identity with the corresponding 5′ end of the rat R2 cDNA (GenBank accession no. AF074482). The 3′ primer (R2-B2 in Supplemental Table 1) was designed based on the untranslated region of the mouse gene. Internal primers (R2-A2 and R2-B1 in Supplemental Table 1) hybridized to protein coding segments that are conserved between the mouse gene as well as the rat and human cDNAs. After PCR amplification, two overlapping segments of cDNA, which together encode the complete R2 protein-coding region, were ligated using a naturally occurring BamHI site and then subcloned into pcDNA1.1. The nucleotide sequence of the protein-coding region of each receptor cDNA was confirmed using automated DNA sequencing (model 373; Applied Biosystems, Foster City, CA).

Generation of Mutant Receptors. R1 and R2 mutations were introduced into the corresponding cDNA using oligonucleotide-directed site-specific mutagenesis as described previously (Beinborn et al., 1993; Blaker et al., 1998). The nucleotide sequence of the protein-coding region of each mutant receptor cDNA was confirmed using automated DNA sequencing.

Luciferase Reporter Gene Assays. Human embryonic kidney (HEK) 293 cells were plated (5000–7000/well) onto 96-well Primaria plates (BD Biosciences, Bedford, MA). After an overnight incubation, cells were transiently transfected with cDNAs encoding each of the following (unless otherwise noted): R1, R2, G_{q5i}, and an SRE_{5x} luciferase reporter gene construct. As reported previously, G_{q5i} enables $G_{i/o}$-coupled receptor activity to be detected using an SRE-luciferase reporter gene (Feuerbach et al., 2000; Hearn et al., 2002). Transfections were done with Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were incubated in serum-free Dubecco's modified Eagle's medium (Invitrogen) either in the absence (for determination of basal activity) or presence of ligand. After overnight stimulation, cells were lysed and luciferase activity was quantified using the LucLite luminescence reporter gene assay system (PerkinElmer Life and Analytical Sciences, Boston, MA). $GABA_BR$ ligands that were studied included CGP54626, CGP52432, CGP55845, CGP35348 (Tocris Cookson Inc., Bristol, UK) as well as baclofen and GABA (Sigma-Aldrich, St. Louis, MO).

Radioligand Binding Experiments. Binding assays were performed as described previously (Lee et al., 1993) with minor modifications. COS-7 cells (10^6/10-cm plate) were transfected using the DEAE dextran method with 5 μg of each relevant $GABA_BR$ subunit cDNA. After ~16 h, cells were split into 24-well plates (80,000 cells/well). The following day, cells were incubated for 80 min at room temperature with 20 nM [^{3}H]CGP54626 (Tocris Cookson Inc.) in the presence of increasing concentrations of unlabeled GABA or CGP54626. Nonspecific binding was assessed in parallel using cells transfected with the empty expression vector pcDNA1.1.

Data Analysis. Ligand concentration-response and competition curves were analyzed using GraphPad Prism software version 3.0 (GraphPad Software Inc., San Diego, CA). All analyses were based on at least three independent experiments. Statistical comparisons were made using InStat software version 3.01 (GraphPad Software Inc.) to calculate either analysis of variance (Dunnett's post tests) or unpaired *t*-tests (two-tailed *p* values).

Results

Cloning and Functional Assessment of the Mouse R1 and R2 Subunits. The cloned R1 sequence that we obtained by PCR is in full agreement with the previously reported cDNA (GenBank accession nos. AF008649 and AF114168).

In addition to the R1 subunit, we cloned R2 using reverse transcription-PCR. Based on computational analysis of the corresponding gene, the mouse R2 cDNA sequence had been predicted to encode a 1455-amino acid protein (GenBank accession no. XM143750). In the current study, we have experimentally established that the actual cDNA encodes a 940-amino acid protein. The discrepancy between the computationally annotated and the cloned mouse R2 cDNA sequence can be attributed to mistaken inclusion of intron segments in the computationally predicted transcript. The cloned mouse R2 cDNA shows a high degree of conservation with other mammalian homologs. The encoded R2 protein is 99.6 and 98% identical to the rat and human R2 subunits, respectively (Supplemental Fig. 1).

As with all known species homologs of this receptor, the mouse $GABA_BR$ functions as a heterodimer composed of an R1 and an R2 subunit. GABA-induced signaling was observed only in cells expressing both $GABA_B$ receptor subunits. In the absence of corresponding heterodimeric partners, basal activity of either the R1 or the R2 subunits approached zero. Note that as reported for the rat homolog (Grunewald et al., 2002), the wild-type mouse $GABA_BR$ shows an appreciable level of basal function (Fig. 1).

A Single Amino Acid Substitution in Either R1 or R2 Results in Constitutive Activity. Within each class of GPCR (A, B, as well as C), naturally occurring activating mutations have been identified and shown to underlie the development of receptor-specific disease (Seifert and Wenzel-Seifert, 2002). Hints about promising candidate domains for mutation-induced receptor activation can often be derived by aligning a target receptor with a related GPCR in which naturally occurring constitutively active variants are known. Using this approach, the $GABA_B$ R1 and R2 amino acid sequences were aligned with constitutively active variants of the calcium sensing receptor (known to result in hypocalcemia) (Jensen et al., 2000) to identify candidate domains for mutagenesis. Based on this rationale, we introduced a series of potential activating mutations into both the R1 and R2 subunits (Table 1). Analysis of these constructs led to the identification of a residue in R2, Tyr690, where four different substitutions resulted in enhanced basal signaling (when each R2 mutant was coexpressed with wild-type R1; Table 1). Among the constitutively active R2 mutants (Table 1), the Y690V variant had the highest level of basal signaling and was therefore selected for further study (Figs. 1 and 2). It is of note that expression of R2 (Y690V), even in the absence of R1, showed a low yet detectable level of constitutive activity (Fig. 1). However, like the wild-type R2 subunit, R2 (Y690V) in the absence of R1 does not respond to GABA.

Although targeted mutagenesis of R2 was successful in identifying a constitutively active variant, a parallel approach with the R1 subunit did not confer ligand-independent signaling (Table 1). Further mutagenesis of residues within the R1 VFTM revealed that a T290K substitution resulted in constitutive activity when the mutant was coexpressed with the wild-type R2 subunit (Figs. 1 and 2). Replacement of Thr290 with Arg, another basic residue also led to an elevation in basal signaling. In contrast, substitution with Leu, a neutral residue, did not induce constitutive activity.

When the most active isoforms of each $GABA_BR$ subunit,

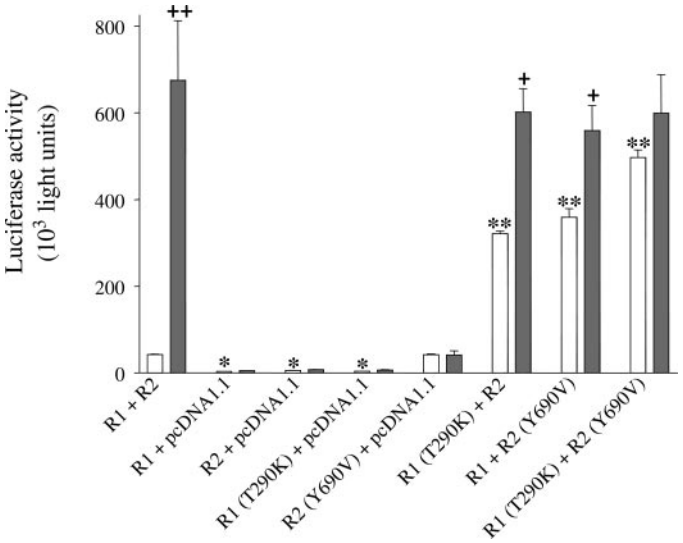

Fig. 1. Single amino acid substitutions in $GABA_BR$ confer ligand-independent signaling. HEK293 cells were cotransfected with cDNAs encoding 1) one or both $GABA_BR$ subunits, 2) G_{q5i}, and 3) an SRE_{5x}-luciferase construct. After overnight stimulation in the absence (open columns) or presence (closed columns) of GABA (10^{-4} M), luciferase activity was quantified. Data represent the mean ± S.E.M. from at least three independent experiments, each performed in triplicate. Basal values were examined for significance relative to that of the wild-type heterodimer (R1 + R2) using analysis of variance followed by Dunnett's post test (*, $p < 0.05$ and **, $p < 0.01$). Agonist-stimulated values were compared with the corresponding basal values for a given receptor by unpaired t test (+, $p < 0.05$ and ++, $p < 0.001$).

TABLE 1

Mutations introduced into the $GABA_BR$ subunits that correspond to constitutively active CaSR variants

Targeted residues in the R1 and R2 subunits were selected by alignment with the human CaSR.

	Known CaSR Activating Mutation	Receptor Domain	Constitutive Activity[a] ($GABA_BR$)
Substitution in R1			
E165K	E191K	N terminal	No
LWLL 666 HPAF[b]	Q681H	TM3	No
L769R	L773R	TM5	No
AV 804 SITF[b]	F806S	TM6	No
A803S	F806S	TM6	No
Substitution in R2			
IWLGI 656 MARGF[b]	L773R	TM5	No
L656R	L773R	TM5	No
Y690V	F806S	TM6	Yes
Y690C	F806S	TM6	Yes
Y690A	F806S	TM6	Yes
Y690G	F806S	TM6	Yes
Y690S	F806S	TM6	No
Y690Q	F806S	TM6	No
Y690E	F806S	TM6	No
Y690N	F806S	TM6	No
Y690W	F806S	TM6	No
Y690K	F806S	TM6	No

[a] Mutants were assessed for constitutive activity in combination with the complementary $GABA_BR$ wild-type subunit.

[b] A block of amino acids in R1 or R2 that were substituted with corresponding amino acids from the CaSR. The corresponding single amino acid activating mutation within the CaSR sequence is underlined.

R1 (T290K) and R2 (Y690V), were coexpressed, an even higher level of basal signaling was observed. It is noteworthy that despite the marked differences in constitutive activity among the wild-type/mutant heterodimer combinations, the maximum level of GABA-mediated signaling remained comparable (Fig. 1).

GABA and Baclofen Potency Are Increased at the Constitutively Active Receptors. To explore the response of the constitutively active R1 and R2 mutants to agonists, HEK293 cells expressing different heterodimer combinations were stimulated with increasing concentrations of GABA or baclofen (Figs. 3 and 4). When either the R1 (T290K) or the R2 (Y690V) subunit was coexpressed with the complementary wild-type subunit, the mutant heterodimer showed a significantly lower EC_{50} for GABA versus the wild-type value (Table 2). Similar potency shifts were observed using baclofen as the agonist. In addition, when both mutants R1 (T290K) and R2 (Y690V) were coexpressed, baclofen potency was further increased. Taken together, our results suggest a trend; increased basal activity is associated with higher agonist potency.

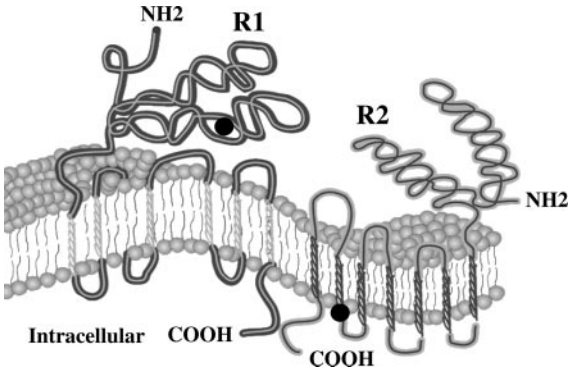

Fig. 2. Cartoon of the $GABA_BR$ illustrating the domains where activating mutations are localized. Filled black circles represent 1) the T290K substitution in the VFTM of R1 and 2) the Y690V mutation in the transition region between transmembrane domain VI and the third intracellular loop of R2. This figure is reproduced with the permission of Sigma-Aldrich.

The High-Affinity Putative Antagonists CGP54626, CGP52432, and CGP55845 Function as Inverse Agonists at the Constitutively Active Receptors. Several compounds that were previously described as high-affinity $GABA_BR$ antagonists were tested for activity on each of the constitutively active mutants (Fig. 3). HEK293 cells expressing combinations of wild-type and/or constitutively active $GABA_BR$ mutant subunits were incubated with increasing concentrations of CGP54626, CGP52432, or CGP55845 (Fig. 5). Each of these compounds attenuated basal signaling of the constitutively active receptors and was thus classified as an inverse agonist. We found a trend suggesting a reciprocal relationship between constitutive receptor activity and potency of each inverse agonist. A statistically significant decrease in potency was found for CGP52432 and CGP55845 when assessed at the double mutant heterodimer, R1 (T290K)/R2 (Y690V) versus the wild-type receptor (Table 2).

CGP35348 Shows Partial Agonist Activity at the Constitutively Active $GABA_B$ Receptors. CGP35348 is described in the literature as a low-affinity antagonist at the wild-type $GABA_BR$. Using the luciferase reporter gene assay, assessment of this compound at the wild-type $GABA_BR$ revealed trace partial agonist activity. At the constitutively active $GABA_BR$ mutants, the efficacy of CGP35348 was more readily detectable (Fig. 6). As observed with GABA and baclofen, there was a trend toward higher potency of CGP35348 with increasing constitutive receptor activity (Table 2). The increase in CGP35348 reached significance when assessed at the double mutant heterodimer R1 (T290K)/R2 (Y690K).

Ligand Binding Assays with Radiolabeled CGP54626. To evaluate the effect of the activating mutations on ligand affinities, we performed radioligand competition binding assays on COS-7 cells expressing either the wild-type $GABA_B$ receptor (R1/R2) or one of three mutant heterodimers, R1/R2 (Y690V), R1 (T290K)/R2, or R1 (T290K)/R2 (Y690V) (Fig. 7). The double mutant heterodimer showed a >100-fold increase in GABA affinity compared with the wild-type receptor. Expression of either R1 (T290K) or R2 (Y690V) with the complementary wild-type subunit showed a 20- or a 12-fold increase in GABA affinity, respectively, com-

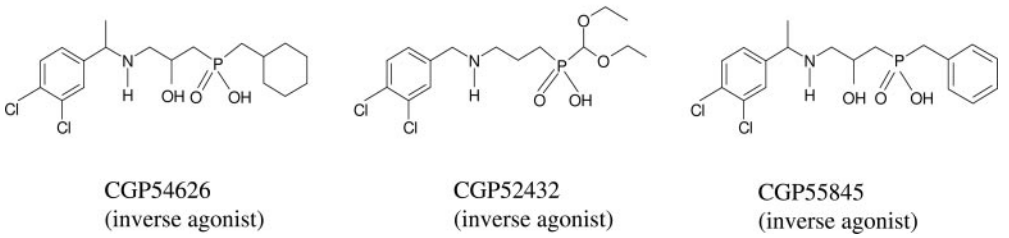

Fig. 3. Structural comparison of the compounds used in this study.

pared with the wild-type heterodimer. Homologous competition binding experiments using radiolabeled versus unlabeled CGP54626 were used to assess the density of binding sites on cells expressing the different $GABA_BR$ constructs. Corresponding values (mean ± S.E.M; $n = 4$) were as follows: wild-type $GABA_BR$ (R1/R2), 2.73 ± 0.65 fmol/10^3 cells; R1/R2 (Y690V), 2.83 ± 0.63 fmol/10^3 cells; R1 (T290K)/R2, 2.56 ± 0.55 fmol/10^3 cells; and R1 (T290K)/R2 (Y690V), 2.73 ± 0.63 fmol/10^3 cells (no significant difference by analysis of variance).

Discussion

The present study led to the novel observation that a single amino acid substitution in either of the two $GABA_BR$ subunits can result in agonist-independent signaling by a heterodimeric GPCR. The availability of these receptor constructs offers the opportunity to investigate agonist versus mutation-induced activation of a class C receptor. The $GABA_BR$ is of additional interest given that this receptor is the prototype obligate heterodimer within the GPCR superfamily (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999) and represents a key target for drug development in the therapeutic areas of human epilepsy, pain, and spasticity.

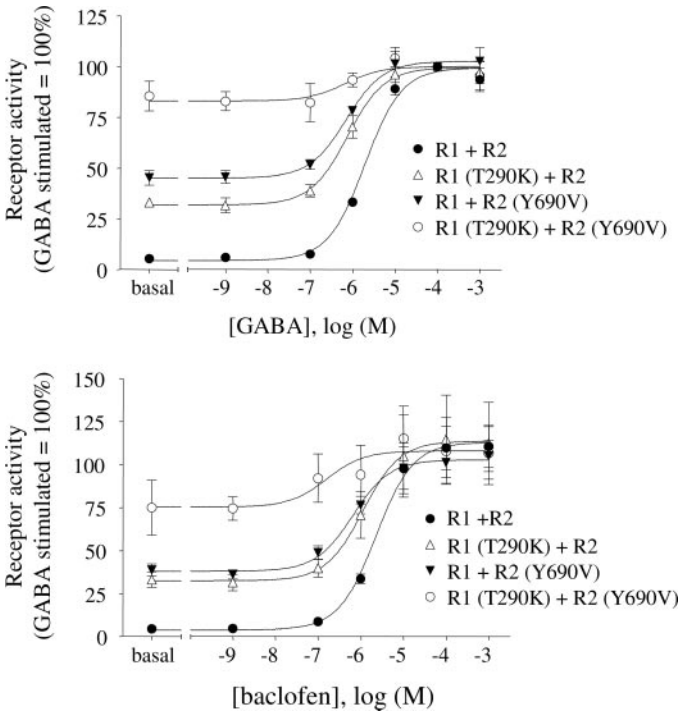

Fig. 4. Constitutively active mutants show higher potency for GABA and baclofen than the corresponding wild-type receptor. Luciferase activity was assessed in HEK293 cells expressing different $GABA_B$ receptor heterodimers, composed of wild-type or mutant subunits as indicated (for methodological details, see Fig. 1 legend). After overnight stimulation with increasing concentrations of GABA or baclofen, luciferase activity was quantified and expressed as a percentage of the maximum GABA-induced effect (after stimulation with 10^{-4} M GABA). Data represent the mean ± S.E.M. from at least four independent experiments, each performed in triplicate (corresponding EC_{50} values are listed in Table 2). "Basal" indicates the absence of ligand.

Much has been learned about $GABA_BR$ function by comparison with the related class C metabotropic glutamate receptor mGluR1. Crystallographic studies of this homodimeric receptor revealed that the N-terminal extracellular region of each subunit includes a bilobate domain (VFTM), which is homologous to bacterial periplasmic proteins (O'Hara et al., 1993; Kunishima et al., 2000; Tsuchiya et al., 2002). Current evidence from mutagenesis studies suggests that agonist stimulation triggers at least one bilobate domain to close. This results in a conformational change in other parts of the receptor (i.e., the helical domain), which in turn activates G proteins. The role of the VFTM, as first described for the mGluR1, is thought to be conserved in other class C GPCRs, including the $GABA_BR$.

We observed that a mutation in the $GABA_BR1$ VFTM (T290K) resulted in constitutive activity that was appreciable only when this subunit was coexpressed with wild-type R2. This may indicate that the N-terminal residue substitution in R1 induces a conformational change similar to that induced by GABA binding and thus results in activation of second messenger signaling through R2. Because R2 does not bind GABA, it would be anticipated, based on current models, that a homologous mutation in the VFTM of the R2 subunit would not trigger constitutive activity. Consistent with this expectation, the corresponding mutation within the R2 subunit (N179K) did not induce ligand-independent signaling (Supplemental Fig. 2).

Much has been learned about the ligand binding mechanism of class C receptors by comparison with the metabotropic glutamate receptor. The crystal structure of the glutamate-bound form of mGluR1 revealed key residues at the interface of the two lobes of the VFTM that are involved in glutamate binding (O'Hara et al., 1993; Kunishima et al., 2000; Tsuchiya et al., 2002). Current evidence suggests that agonist stimulation of mGlu1R triggers VFTM closure. Among the residues that comprise the glutamate binding pocket is Asp208. Homology modeling suggests that the corresponding residue in the mouse $GABA_B$ R1 is Ala291

TABLE 2
Ligand potencies at the wild-type and mutant $GABA_B$ receptors

Ligand	R1 + R2		R1 (T290K) + R2			R1 + R2 (Y690V)			R1 (290K) + R2 (Y690V)		
	EC_{50}[a]	pEC_{50} ± S.E.M.	EC_{50}[a]	pEC_{50} ± S.E.M.	Shift[b]	EC_{50}[a]	pEC_{50} ± S.E.M.	Shift[b]	EC_{50}[a]	pEC_{50} ± S.E.M.	Shift[b]
GABA	1960	5.71 ± 0.01	665	6.18 ± 0.10[c]	2.9	600	6.22 ± 0.10[c]	3.2	723	6.14 ± 0.15[d]	2.7
Baclofen	2230	5.65 ± 0.06	1047	5.98 ± 0.10	2.1	596	6.22 ± 0.17	3.7	90.6	7.04 ± 0.59[d]	25
CGP54626	4.9	8.31 ± 0.23	3.4	8.46 ± 0.15	1.4	4.5	8.35 ± 0.15	1.1	20.9	7.68 ± 0.08	0.2
CGP52432	38.0	7.42 ± 0.14	36.9	7.43 ± 0.15	1.0	78.9	7.10 ± 0.10	0.5	419.8	6.37 ± 0.12[c]	0.1
CGP55845	1.7	8.78 ± 0.29	3.7	8.43 ± 0.08	0.4	8.0	8.09 ± 0.22	0.2	27.4	7.56 ± 0.13[c]	0.1
CGP35348	14,490	4.84 ± 0.21	17180	4.76 ± 0.21	0.8	5320	5.27 ± 0.14	2.8	912	6.04 ± 0.40[d]	16.7

[a] Half-maximal effective concentration (nanomolar), average of at least three independent experiments.
[b] -Fold shift in EC_{50}, calculated as ratio of EC_{50} (wild type)/EC_{50} (mutant).
[c] $p < 0.01$ versus wild-type value.
[d] $p < 0.05$ versus wild-type value.

(Kniazeff et al., 2002). In our study, we demonstrate that mutation of the adjacent R1 amino acid, T290K, results in ligand-independent signaling. We speculate that the positively charged lysine introduced in place of Thr290 interacts with one or more negatively charged residue(s) within or proximal to the putative ligand pocket, thereby inducing closure of the venus fly trap. This proposed mechanism is reminiscent of the previously reported constitutive receptor activation resulting from closure of the VFTM due to introduction of a disulfide bridge in this domain (Kniazeff et al., 2004). In contrast to the constitutively active mutants described in the current study, which maintain agonist responsiveness, covalent modification of the receptor abolished ligand-induced function.

Mutation of the R2 subunit Y690V also resulted in constitutive activity. The Y690V substitution is located at the transition between transmembrane domain VI and the third intracellular loop, a putative G protein binding domain (Duthey et al., 2002). The location of this activating substitution raises the possibility that constitutive activity results from a mutation induced change in the interaction between $GABA_BR$ and the G protein. In addition, our findings provide independent support for the postulated mechanism of $GABA_BR$-mediated signaling, in which G protein coupling occurs through the R2 subunit (Robbins et al., 2001; Duthey et al., 2002; Havlickova et al., 2002). Consistent with this model, the homologous mutation in R1 (A803V) does not induce ligand-independent signaling (Supplemental Fig. 2).

In contrast to R1 (T290K), which exhibits no activity in the absence of R2, the R2 variant Y690V shows a low level of constitutive activity even when expressed alone. This observation is in accordance with the established ability of R2 to independently reach the cell surface and confer G protein coupling (Binet et al., 2004; Pin et al., 2004). Our finding that constitutive activity of R2 (Y690V) is greatly enhanced with coexpression of wild-type R1 suggests that the latter subunit

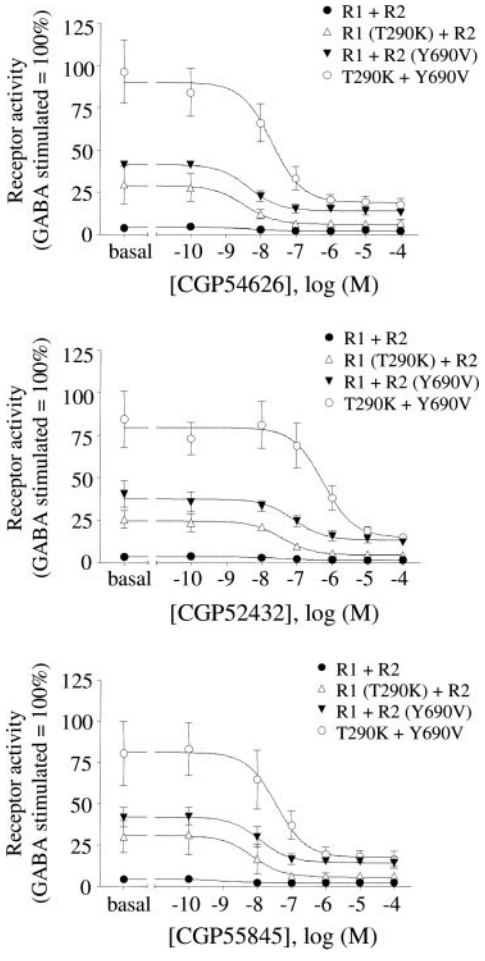

Fig. 5. Putative high-affinity antagonists CGP52432, CGP54626, and CGP55845 function as inverse agonists at each of the constitutively active receptors. Luciferase activity was assessed in HEK293 cells expressing different $GABA_B$ receptor heterodimers, composed of wild-type or mutant subunits as indicated (for methodological details, see Fig. 1 legend). After overnight stimulation with increasing concentrations of CGP54626, CGP52432, or CGP55845, luciferase activity was quantified and expressed as a percentage of the maximum GABA-induced effect (after stimulation with 10^{-4} M GABA). Data represent the mean ± S.E.M. from at least three independent experiments, each performed in triplicate (corresponding EC_{50} values are listed in Table 2). Basal indicates signaling in the absence of ligand.

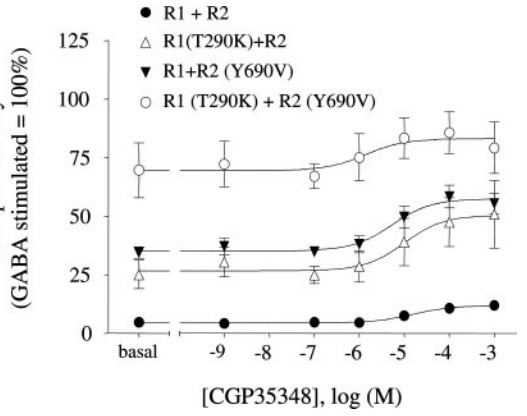

Fig. 6. CGP35348, a putative low-affinity antagonist, shows weak partial agonist activity. Luciferase activity was assessed in HEK293 cells expressing different $GABA_B$ receptor heterodimers, composed of wild-type or mutant subunits as indicated (for methodological details, see Fig. 1 legend). After overnight stimulation with increasing concentrations of CGP35348, luciferase activity was quantified and expressed as a percentage of the maximum GABA-induced effect (after stimulation with 10^{-4} M GABA). Data represent the mean ± S.E.M. from at least three independent experiments, each performed in triplicate (corresponding EC_{50} values are listed in Table 2). Basal indicates absence of ligand.

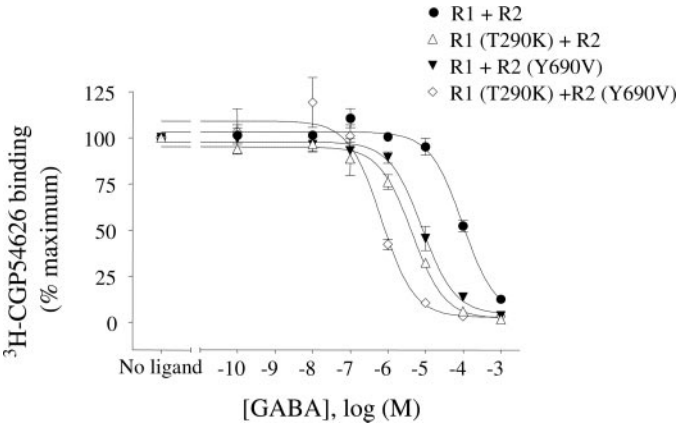

Fig. 7. Constitutively active $GABA_BR$ mutants show higher affinity for GABA than the wild-type heterodimer. COS-7 cells were cotransfected with different $GABA_B$ receptor heterodimers, composed of wild-type or mutant subunits as indicated. Cells were incubated with [^{3}H]CGP54626 in the presence of increasing concentrations of unlabeled competitor, GABA. Mean IC_{50} values were as follows: R1 + R2, 97700 nM; R1 (T290K) + R2, 5010 nM; R1 + R2 (Y690V), 7940 nM; and R1 (T290K) + R2 (Y690V), 695 nM.

acts as a positive modulator of R2-mediated signaling, in addition to its well established role as a GABA binding site. In fact, our observation provides independent support for the postulated role of R1 as an enhancer of R2 coupling efficiency. The concept that R1 acts as a modulator of R2 function was originally proposed to explain unexpected pharmacological properties of chimeric $GABA_BR$ heterodimers. These constructs were engineered to include only one type (either R1 or R2) of helical domain (Galvez et al., 2001; Kniazeff et al., 2004; Pin et al., 2004). Study of these chimeric receptors demonstrated that absence of the R1 helical domain markedly reduced the efficacy and potency of GABA (Galvez et al., 2001). These findings, together with our observation that constitutive activity of a variant R2 is markedly enhanced by R1, suggest that $GABA_BR$ signaling, in the presence or absence of ligand is enabled by an interplay between the helical domains of both subunits.

In addition to the point mutations reported in this study, two other $GABA_BR$ modifications have been shown to result in ligand-independent signaling. In one report, it was demonstrated that chimeric $GABA_BR$ heterodimers, which include only one type of extracellular domain (either R1 or R2), show a modest degree of basal signaling. Such receptors, however, no longer respond to GABA (Galvez et al., 2001; Pin et al., 2004). A second report describes a constitutively active $GABA_BR$ heterodimer that resulted from introduction of two cysteine residues within the VFTM of R1 (Kniazeff et al., 2004). These residues form a putative disulfide bridge, thus locking the binding domain in a closed position and triggering downstream signaling. Supporting this interpretation, constitutive activity can be inhibited by the reducing agent dithiothreitol.

As opposed to the construct described above, constitutive activity of the mutant receptors R1 (T290K) and R2 (Y690V), as reported here, does not require covalent modification of either $GABA_BR$ subunit. In the latter variants, the single amino acid substitutions that induce constitutive activity seem to conserve the receptor's ability to undergo transitions between "active" and "inactive" states. In contrast to the properties of the previously reported chimeras and cysteine-substituted mutants, ligand binding and agonist-induced function of the R1 (T290K) and R2 (Y690V) receptors remain intact, enabling further studies of the pharmacological changes associated with enhanced basal receptor function.

The current view of GPCR activation suggests that either mutations or agonists can preferentially stabilize the active conformation of a receptor over the inactive state (Samama et al., 1993), thus triggering G protein activation. Conversely, an inverse agonist stabilizes the inactive form of the receptor, thereby decreasing basal receptor signaling. This model was originally proposed based on studies with a constitutively active β-adrenoceptor mutant (Samama et al., 1993; Leff, 1995); its applicability to many other class A GPCRs has subsequently been shown. Together, these experiments have revealed certain characteristic features of constitutively active receptors that support the model, and, at the same time, provide a basis for comparison with structurally distinct receptors (i.e., class C heterodimers).

Constitutively active class A receptors, compared with corresponding wild-type receptor proteins, 1) show an increase in agonist potency and affinity, 2) distinguish inverse agonists from antagonists, and 3) have conserved antagonist affinity (Samama et al., 1993; Tiberi and Caron, 1994; Beinborn et al., 1998). Our results suggest that these features can be extrapolated to class C $GABA_B$ receptors, despite their minimal sequence homology with class A receptors and their dependence on heterodimerization as a prerequisite of function. Consistent with the current model of GPCR activation (Lefkowitz et al., 1993; Kenakin, 2002), activating mutations in the $GABA_BR$ that increase basal signaling also enhance agonist potency and affinity (Figs. 4 and 7; Table 2). These pharmacological alterations were observed with point mutations in either $GABA_BR$ subunit when coexpressed with the complementary wild-type subunit. In addition, coexpression of the two constitutively active subunits resulted in even more pronounced shifts compared with the wild-type heterodimer.

Another pharmacological feature that broadly applies to constitutively active receptors is the ability to inhibit ligand-independent signaling with inverse agonists (Samama et al., 1993; Tiberi and Caron, 1994; Beinborn et al., 1998). In the current study, we found that two structurally related compounds, CGP52432 and CGP55845, previously considered as neutral antagonists, inhibited basal signaling of the mutant $GABA_BR$ and thus should be classified as inverse agonists. In addition, using $GABA_BR$ mutants with significant basal activity, we were able to confirm the classification of CGP54626 as an inverse agonist. A previous report suggested that CGP54626 had inverse agonist activity when assessed at the rat wild-type $GABA_BR$; like the wild-type mouse receptor, the rat homolog shows an appreciable level of ligand-independent signaling (Grunewald et al., 2002). All three compounds (CGP54626, CGP52432, and CGP55845) share relatively high $GABA_BR$ binding affinity. It is of note that the potency of the three inverse agonists tends to decrease as the level of constitutive activity increases (i.e., as a shift occurs from the inactive to the active receptor state). This is consistent with current models in which the inactive conformation is most favorable to inverse agonist interactions, whereas agonists preferentially bind the active form of the receptor (Gether, 2000).

Constitutively active mutants may be used to detect ligand activity that is not evident at the wild-type receptor (Fig. 6). Once a ligand with intrinsic activity (i.e., an agonist or inverse agonist) is identified, it may be anticipated that structural modification can lead to the identification of full agonists. In a previous manuscript, our group illustrated this strategy with the CCK-2 receptor, a class A GPCR. Reminiscent of our current findings with the $GABA_BR$, we showed that putative "antagonists" of the wild-type CCK receptor actually had significant partial or inverse agonist activity when tested on a constitutively active CCK-2 receptor mutant. Screening of a series of structural derivatives of these compounds led to the identification of molecules with near full agonist activity, both in vitro and in vivo (Kopin et al., 2003). Based on these prior observations, it will be of interest to examine the activity of structural derivatives of the partial/inverse agonists identified using the constitutively active $GABA_B$ receptors. This approach provides a potential means to expedite the identification of novel, therapeutically useful $GABA_BR$ drugs.

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