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Pharmacological Modulation of the Diazepam-Insensitive Recombinant γ-Aminobutyric Acid$_A$ Receptors α4β2γ2 and α6β2γ2

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SUMMARY

We characterized modulation of the γ-aminobutyric acid (GABA)-evoked responses of the diazepam-insensitive α4β2γ2 and α6β2γ2 recombinant GABA$_A$ receptors. The partial agonist bretazenil potentiated the responses of both receptors with similar dose dependence but with a higher maximal enhancement at the α4β2γ2 receptor. The bretazenil-induced potentiation was reduced by the benzodiazepine antagonist flumazenil. At a high concentration (10 μM), flumazenil was a weak potentiator of the GABA response. The partial agonist imidazenil was inactive. The imidazobenzodiazepine inverse agonist Ro 15–4513, which is known to bind with high affinity to the α6β2γ2 receptor, potentiated the GABA responses of the α4β2γ2 and α6β2γ2 receptor subtypes with similar dose dependence over the concentration range of 0.1–10 μM. Methyl-6,7-dimethoxy-4-ethyl-β-carboline, a β-carboline inverse agonist, had a similar potentiating effect when tested at a concentration of 10 μM. The α4β2γ2 and α6β2γ2 receptor-mediated currents had equal sensitivities to furosemide and Zn^{2+} ions, both of which reduced the GABA-evoked responses. The α6β2γ2 receptor but not the α4β2γ2 receptor exhibited a low level of spontaneous activity in the absence of GABA; this resting current could be directly potentiated by Ro 15–4513, methyl-6,7-dimethoxy-4-ethyl-β-carboline, bretazenil and flumazenil and was blocked by picrotoxin. Thus, although the α4β2γ2 and α6β2γ2 receptors are insensitive to benzodiazepine binding site full agonists, such as diazepam, they can be modulated by certain ligands acting as partial and inverse agonists at diazepam-sensitive receptors and thereby contribute to the respective pharmacological profiles.

GABA$_A$ receptors, which mediate much of the inhibitory synaptic transmission in the mammalian brain, consist of a pseudosymmetrical, pentameric array of transmembrane subunits forming the GABA-gated Cl^- ion channel. Based on a repertoire of five classes of subunits (α1-6, β1-3, γ1-3, ρ1, ρ2, and δ), the molecular architecture of the receptor is extremely heterogeneous, although it comprises, in most cases, combinations of α, β, and γ subunits. GABA$_A$ receptors possess binding sites for several classes of modulatory compounds, including the ligands that act at the benzodiazepine-binding site. The type of α subunit (i.e., α1, α2, α3, α5) plays a predominant role in fine-tuning the selectivity of the receptor for ligands active at this site (1). In contrast, receptors containing the α6 subunit, in combination with a β1 or β2 subunit and a γ2 subunit, are insensitive to the classic benzodiazepine agonists diazepam and flunitrazepam but exhibit high affinity for the imidazobenzodiazepine inverse agonist Ro 15–4513 (2). For α4-containing recombinant receptors, binding studies have also shown insensitivity to diazepam and high affinity for Ro 15–4513 (3).

Diazepam-insensitive GABA$_A$ receptors that bind Ro 15–4513 occur in the brain, particularly in the cerebellar granule cells (4), for which immunocytochemical studies suggest a predominance of α6, β2, and γ2 subunits (5, 6). There is evidence suggesting that the α6 subunit in large part replaces the α1 as the predominant α subunit in GABA$_A$ receptors of the cerebellar granule cells during the first 20 days *in vitro* (7), and this seems to reflect the changes in α6 mRNA level and diazepam sensitivity that take place during development of the cerebellum *in vivo* (8, 9). In contrast, the

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ABBREVIATIONS: GABA, γ-aminobutyric acid; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/Tween 20; DMSO, dimethylsulfoxide.

$\alpha4\beta2\gamma2$ receptor is rare in the cerebellum but highly expressed in the thalamus and at lower levels in cortex and putamen, as shown by *in situ* hybridization studies (3). The contribution of these receptors to the pharmacological profile of $GABA_A$ receptor modulators has been only partly clarified.

The $\alpha6$ subunit-containing receptors are modulated by loreclezole (10) and neurosteroids (11). In addition, certain ligands, such as pyrazinones and β-carbolines, that act at classic benzodiazepine sites also interact with the $\alpha6$ subunit-containing receptors (12–14). *In situ*, moderate-to-high binding affinities at the [^{3}H]Ro 15–4513-binding site on diazepam-insensitive cerebellar $GABA_A$ receptors have been reported for several structurally diverse compounds (15). However, it was not known whether ligands that act as partial agonists at classic diazepam-binding sites also act on the diazepam-insensitive $\alpha4$ and $\alpha6$ subunit-containing receptors. Therefore, the modes of action of the partial agonists bretazenil and imidazenil were tested in comparison with the inverse agonists Ro 15–4513 and DMCM on the diazepam-insensitive recombinant $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ $GABA_A$ receptors by radioligand binding and electrophysiological analysis. Furosemide, which has been suggested to be the first antagonist specific for $\alpha6\beta2\gamma2$ and $\alpha6\beta3\gamma2$ receptors (16), and Zn^{2+} ions were also included in this study.

Materials and Methods

Cell culture and transfection. A stable cell line expressing the $\alpha6$, $\beta2$, and $\gamma2$(short) subunits of rat $GABA_A$ receptors was derived by transfection of plasmids containing the subunit cDNAs and plasmids encoding geneticin resistance into HEK 293 cells, as described elsewhere (17). To transiently transfect HEK 293 cells with the rat cDNA combinations $\alpha4\beta2\gamma2$ (3) and $\alpha6\beta2\gamma2$ (2, 18), the calcium phosphate precipitation technique (19) was used, as described previously (20). The cells were grown in minimal essential medium (GIBCO, Paisley, Scotland) supplemented with 8% fetal calf serum (GIBCO) and 50 mg/ml gentamycin (GIBCO). The culture medium for the $\alpha6\beta2\gamma2$ stable cell line contained, in addition, 200 mg/ml geneticin (GIBCO). For the electrophysiological experiments, the cells were seeded onto glass coverslips coated with fibronectin (Sigma, Buchs, Switzerland).

Membrane preparation and radioligand-binding assay. Cells from 10–20 culture dishes (15 cm) were harvested and collected by centrifugation for 15 min at 10,000 × *g*. The cells were homogenized in 10 volumes of 5 mM Tris·HCl, pH 7.4, 5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride and centrifuged at 40,000 × *g* for 15 min. After being washed three times with buffer by resuspension and centrifugation, the resulting pellet was resuspended in buffer to give a final protein concentration of 5–10 mg/ml.

For Scatchard analysis, aliquots of the homogenate (0.2–0.5 mg of protein) were incubated with increasing concentrations of [^{3}H]Ro 15–4513 (1–40 nM) in a total volume of 0.2 ml for 90 min on ice. After termination of the reaction by the addition of 4 ml of buffer and rapid vacuum filtration, the Whatman GF/C filters were washed twice with 4 ml of buffer, and radioactivity was measured by liquid scintillation counting. Nonspecific binding at each [^{3}H]Ro 15–4513 concentration was determined by including 10 μM unlabeled Ro 15–1788 (flumazenil) in parallel incubations. Drug binding profiles were determined with serial dilutions of various drugs at a fixed concentration of [^{3}H]Ro 15–4513 (10 nM). Scatchard analysis and evaluation of the drug displacement data were performed using the LIGAND program (21).

Electrophysiology and data analysis. The whole-cell configuration of the patch-clamp technique was used to record GABA-induced Cl^- currents. Recording pipettes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany) and had resistances of 2–4 MΩ when filled with intracellular solution. The membrane potential was held to −60 mV. The current signals were amplified with an L/M EPC 7 patch-clamp amplifier (List Medical Instruments, Darmstadt-Eberstadt, Germany), filtered by a 60-Hz four-pole Bessel low-pass filter, digitized by a TL-1 Labmaster board (Axon Instruments, Foster City, CA), and stored on a computer (486, 66 MHz). For data analysis, the pClamp data acquisition program set (Axon Instruments), the RS/1 software package (BBN Software Products, Cambridge, MA), and FigP (Biosoft, Cambridge, UK) were used.

The GABA dose-response curves were obtained by applying 2-sec pulses of GABA every 2 min to the patch-clamped HEK 293 cells. The maximum current amplitudes from individual cells were first fitted separately using the equation $I/I_{max} = 1/[1 + (EC_{50}/[GABA])^{n_H}]$, where I is GABA-evoked current, max is the maximum of the fit, EC_{50} is the GABA concentration evoking the half-maximal response, and n_H is the Hill coefficient.

The individual dose-response curves were then normalized to I_{max}, and the mean ± standard error values calculated from the normalized data for each concentration were plotted and fitted with a sigmoidal curve generated to allow the mean EC_{50} and Hill values to be calculated from the dose-response curves of the individual cells.

Drug application. GABA in the presence or absence of drugs was applied to the patch-clamped cell for 2 sec at 2-min intervals using a multibarrelled microapplicator pipette constructed from seven concentrically arranged glass tubes (i.d., 320 μm) ending in a common tip (dead volume, <1 μl). Six tubes were used for drug delivery, and the seventh tube provided slow aspiration to prevent accumulation of drugs in the common tip and leakage from the inactive barrels onto the cell. Complete solution change around the cell was accomplished within 100–300 msec (20). For each experiment, at least three GABA control responses were evoked, and only cells showing stable GABA responses were selected for the drug testing. Before microapplication of a GABA-drug mixture, the same concentration of the drug alone was applied by bath perfusion for 1 min.

Solutions and drugs. The recording pipettes were filled with an intracellular solution containing 140 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM EGTA, and 10 mM HEPES, adjusted to pH 7.2 with CsOH (osmolarity, 306 mOsM). The cells in the recording chamber were continuously superfused with a bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES/NaOH, adjusted to pH 7.4 with NaOH (osmolarity, 296 mOsM). To avoid the occurrence of cell swelling, the intracellular solution was sometimes diluted by 10%. The experiments were carried out at room temperature. Stock solutions of the test compounds were prepared in 100% DMSO and diluted 1000-fold before use. During the experiment, all bath solutions contained 0.1% DMSO (0.2% DMSO in flumazenil competition experiments), which by itself had no detectable effect on the GABA responses. The benzodiazepine ligands bretazenil, Ro 15–4513, diazepam, imidazenil, and flumazenil were kindly provided by Hoffmann-La Roche (Basel, Switzerland), and abecarnil was provided by Schering (Berlin, Germany). [^{3}H]Ro 15–4513 was purchased from Dupont (Regensdorf, Switzerland), and GABA, sodium pentobarbital, picrotoxin, furosemide, and bicuculline methochloride were purchased from Sigma. $ZnSO_4$ was from Fluka (Buchs, Switzerland), and DMCM from Research Biochemicals International (Zurich, Switzerland).

SDS-PAGE and Western blotting. Aliquots of crude membrane preparations were prepared for SDS-PAGE by incubation for 15 min at 60° with an equal volume of 125 mM Tris·HCl, pH 6.8, 20% glycerol, 0.002% bromphenol blue, 10% β-mercaptoethanol, and 4% SDS, followed by centrifugation at 15,000 × *g* for 5 min. The supernatant was subjected to SDS-PAGE using 10% minigels (Mini Protean II, BioRad, Glattbrugg, Switzerland). Proteins were blotted onto nitrocellulose membranes in a semidry electroblotting apparatus (Trans Blot, BioRad) at 15 V for 45 min using 39 mM glycine, 48 mM Tris, 0.04% SDS, and 20% methanol as a transfer buffer. For the immunodetection, the blots were blocked for 2 hr in TBST containing

5% nonfat dry milk (blocker) at room temperature, followed by incubation with the affinity-purified subunit-specific antisera overnight at 4° in the same solution. The blots were washed once for 10 min with 20 mM Tris, pH 7.5, 60 mM NaCl, 2 mM EDTA, 0.4% SDS, 0.4% Triton X-100, 0.4% deoxycholate followed by three washes in TBST. Incubation with secondary antibodies was carried out for 2 hr at room temperature [horseradish peroxidase-conjugated goat anti-rabbit IgG (Promega, Catalys, Wallisellen, Switzerland)] diluted 1:2500 in TBST/5% blocker. After extensive washing (see above), immunoreactivity was detected by the chemiluminescence method (ECL, Amersham International, Zürich, Switzerland) according to the manufacturer's instructions.

Results

Ligand-binding characteristics of the Ro 15–4513-binding site. The recombinant $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ $GABA_A$ receptors used in the current study showed high affinity for [^{3}H]Ro 15–4513 ($\alpha4\beta2\gamma2$, K_D = 7.3 ± 1 nM; $\alpha6\beta2\gamma2$, K_D = 7.0 ± 1 nM) and displayed similar binding site density (in the range of 100–150 fmol/mg of protein). In keeping with previous results (2, 3, 15), the classic benzodiazepine agonists, diazepam and flunitrazepam, were inactive in displacing [^{3}H]Ro 15–4513. However, the partial agonist bretazenil, but not imidazenil, and the classic benzodiazepine antagonist flumazenil were found to bind to the $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors with high nanomolar affinities (Table 1). The effects of these ligands on the GABA responses mediated by the $\alpha4$ and $\alpha6$ subunit-containing receptors were therefore determined electrophysiologically and compared with the effects of the known ligands Ro 15–4513 and DMCM.

GABA-evoked currents. The EC_{50} values and Hill coefficients were comparable for the two $GABA_A$ receptor subtypes (Fig. 1): $\alpha4\beta2\gamma2$, EC_{50} = 3.9 ± 0.7 μM and Hill coefficient = 1.0 ± 0.20 (mean ± standard error, five experiments); $\alpha6\beta2\gamma2$, EC_{50} = 1.4 ± 0.3 μM and Hill coefficient 1.2 ± 0.3 (12 experiments), which is consistent with earlier results (16, 22, 23). For the drug experiments described below, a GABA concentration of 1 μM was chosen, which corresponded to the EC_{20} value for the $\alpha4\beta2\gamma2$ receptor and the EC_{30} value for the $\alpha6\beta2\gamma2$ receptor (Fig. 1) and allowed the possibility of several-fold drug-induced potentiation of the GABA-evoked response.

The current-voltage relation for the GABA-evoked current reversed at a membrane potential (E_{rev} = −5.06 ± 0.34 mV; four experiments; $\alpha6\beta2\gamma2$ receptor) close to the Cl^- equilibrium potential calculated from the Nernst equation (1.2 mV), which was expected for a current mediated by a $GABA_A$ receptor channel. The GABA response was reversibly reduced to 31.3 ± 5.0% (three experiments) by picrotoxin (20 μM, Fig. 2A) and to 11.1 ± 5.1% (three experiments) by bicuculline methochloride (10 μM, Fig. 2B) when tested on the $\alpha6\beta2\gamma2$ receptor. The response was reversibly enhanced to 348.5 ± 56.6% (four experiments) of the control by 100 μM sodium pentobarbital (Fig. 2C).

TABLE 1

[^{3}H]Ro 15-4513 displacement profile for the recombinant $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ $GABA_A$ receptors

Displacer	K_i m ± SD (n = 3–4)	
	$\alpha4\beta2\gamma2$	$\alpha6\beta2\gamma2$
	nM	
Ro 15-4513[a]	7.3 ± 1	7.0 ± 1
Bretazenil	15 ± 5	20 ± 5
Flumazenil	148 ± 19	100 ± 15
DMCM	62 ± 17	487 ± 53
Diazepam	>100,000	>100,000
Imidazenil	>100,000	>100,000

[a] K_D determined by Scatchard analysis.
Values are mean ± standard deviation from three or four experiments.

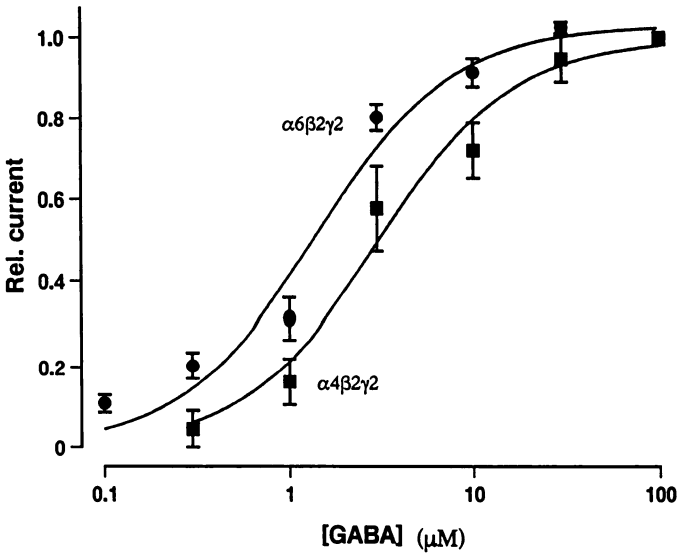

Fig. 1. GABA dose-response curves obtained from HEK 293 cells transiently transfected with cDNAs coding for the $\alpha4\beta2\gamma2$ $GABA_A$ receptor subunits or stably transfected with cDNAs coding for the $\alpha6\beta2\gamma2$ $GABA_A$ receptor subunits. The maximum current amplitudes were measured and plotted as described in Materials and Methods. Values are mean ± standard error for $\alpha4\beta2\gamma2$ (■, five experiments) and $\alpha6\beta2\gamma2$ (●, 12 experiments).

Modulators of the GABA-evoked current. Bretazenil is a partial benzodiazepine agonist for $GABA_A$ receptors containing a β and a γ2 subunit in combination with an α1, α2, α3, or α5 subunit (24, 25). In cells expressing the $\alpha6\beta2\gamma2$ receptor, bretazenil, at 1 μM, potentiated the GABA-evoked response, which is in keeping with its high affinity to the [^{3}H]Ro 15–4513-binding site (Table 1). The GABA-evoked currents mediated by both the $\alpha4\beta2\gamma2$ and the $\alpha6\beta2\gamma2$ receptors were potentiated by bretazenil in a dose-dependent manner (Fig. 3). The EC_{50} values were 311 ± 87 nM for $\alpha4\beta2\gamma2$ (four experiments) and 158 ± 17 nM for $\alpha6\beta2\gamma2$ (eight experiments). The maximal potentiation induced by bretazenil (10 μM) was higher for the $\alpha4\beta2\gamma2$ receptor (310 ± 20% of control, four experiments) than for the $\alpha6\beta2\gamma2$ receptor (169 ± 12% of control, six experiments).

In addition, we tested whether the potentiating effect of bretazenil was sensitive to flumazenil because our binding data show that flumazenil has an affinity to the [^{3}H]Ro 15–4513-binding site only 5–10-fold lower than that of bretazenil on the $\alpha4$ and $\alpha6$ subunit-containing receptors. The potentiating effect of 1 μM bretazenil on the $\alpha6\beta2\gamma2$ receptor was significantly reduced from 155.8 ± 6.3% to 124.3 ± 6.8% (six experiments; $p < 0.01$, two-tailed t test) by flumazenil (1 μM) (Fig. 4). When a higher dose of flumazenil was used (10 μM), the interpretation of the data was complicated by an agonistic effect of flumazenil, which became at that dose (data not shown).

To determine whether partial agonists with benzodiazepine structure might in general be active on the diazepam-

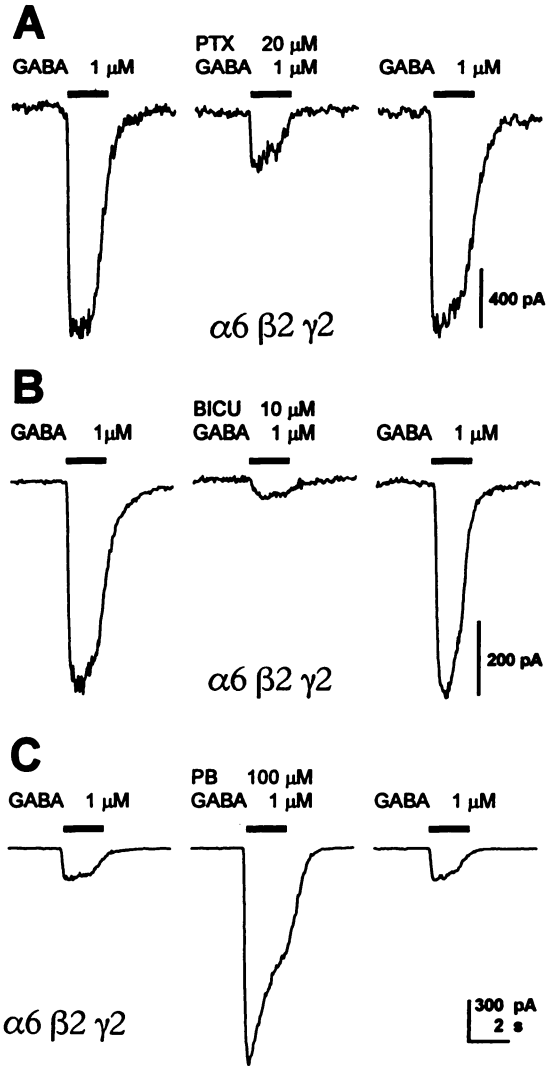

Fig. 2. Effects of picrotoxin, bicuculline, and pentobarbital on the GABA-evoked response of cells stably transfected with the cDNAs coding for the $\alpha6\beta2\gamma2$ subunit combination. GABA (1 μM) was applied at 2-min intervals alone or in the presence of (A) picrotoxin (*PTX*, 20 μM), (B) bicuculline methochloride (*BICU*, 10 μM), or (C) sodium pentobarbital (*PB*, 100 μM) for varying time periods (*bars*). The drugs were preapplied by bath perfusion for ≥1 min before microapplication of the GABA/drug mixture. *Traces* in A–C were recorded from different cells.

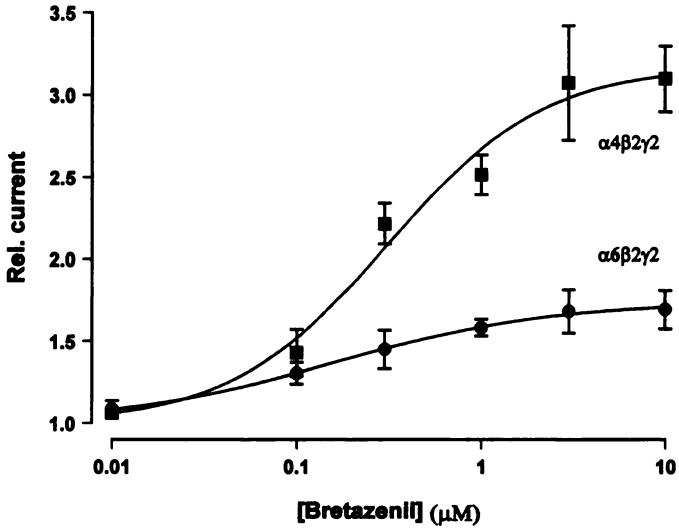

Fig. 3. Dose-response curves for the potentiating effect of bretazenil on the GABA-evoked current mediated by $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ $GABA_A$ recombinant receptors. The current amplitudes obtained from GABA applications (1 μM) in the presence of different concentrations of bretazenil are plotted as a function of the drug concentration. The maximum current amplitudes were measured and plotted as described in Materials and Methods. Values are normalized to the control responses obtained with GABA alone. A relative current of 1.0 represents the GABA-induced current in the absence of bretazenil. Values are mean ± standard error for $\alpha4\beta2\gamma2$ (■, four experiments) and $\alpha6\beta2\gamma2$ (●, nine experiments).

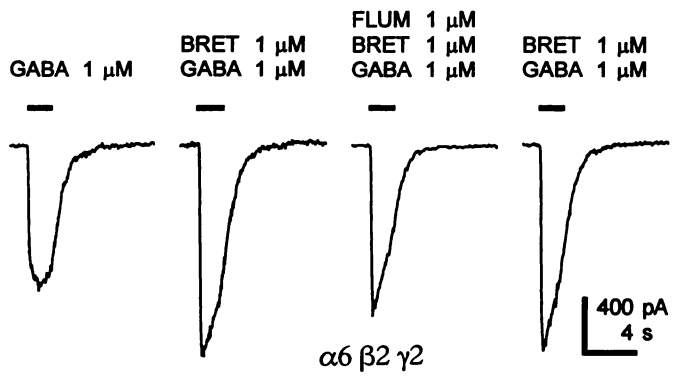

Fig. 4. Antagonism by flumazenil of the potentiating effect of bretazenil on the GABA-evoked response mediated by the $\alpha6\beta2\gamma2$ $GABA_A$ receptor. The GABA-evoked current was potentiated bretazenil (*BRET*, 1 μM), and this potentiation was reversibly decreased by simultaneous application of flumazenil (*FLUM*, 1 μM). *Bars*, periods of drug application.

insensitive receptors, we tested imidazenil, a partial agonist at the benzodiazepine-binding site of $GABA_A$ receptors (26). It had no effect on the $\alpha6\beta2\gamma2$ receptor at a concentration of 10 μM (111.0 ± 7.0% of control; four experiments), as expected from its lack of affinity to the [^{3}H]Ro 15–4513-binding site of this receptor (Table 1).

Consistent with the lack of competitive binding by diazepam at the [^{3}H]Ro 15–4513-binding site (Table 1), diazepam (1 μM) had no effect on the current evoked by 1 μM GABA (98.0 ± 2.0% of control; six experiments) from $\alpha6\beta2\gamma2$ receptor-expressing cells. On the other hand, Ro 15–4513 (1 μM), a partial inverse agonist on receptors displaying classic benzodiazepine pharmacology ($\alpha1\beta2\gamma2$, $\alpha3\beta2\gamma2$, $\alpha5\beta2\gamma2$), enhanced the GABA-evoked response to 140.0 ± 9.0% of control (four experiments). The contrast between the lack of effect of diazepam and potentiation by Ro 15–4513 could be observed in the same cell (Fig. 5). The potentiating effect of Ro 15–4513 was at variance with the results of a previous study in which Ro 15–4513 (1 μM) was reported to reduce rather than enhance the GABA current mediated by recombinant $GABA_A$ receptors composed of rat $\alpha6\beta2\gamma2$ subunits transiently expressed in HEK 293 cells (14). We therefore investigated in detail the mode of action of this compound.

The effect of Ro 15–4513 was tested on both the transiently expressed $\alpha4\beta2\gamma2$ and the stably transfected $\alpha6\beta2\gamma2$ receptors to generate full dose-response curves. Ro 15–4513 potentiated the GABA-induced current at all concentrations tested in a dose-dependent and reversible manner on both the transiently expressed $\alpha4\beta2\gamma2$ and stably transfected $\alpha6\beta2\gamma2$ receptors (Fig. 6). The EC_{50} values for potentiation by Ro 15–4513 were 105 ± 4 nM for $\alpha4\beta2\gamma2$ (four experiments) and 168 ± 65 nM for $\alpha6\beta2\gamma2$ (nine experiments) (Fig. 6). The

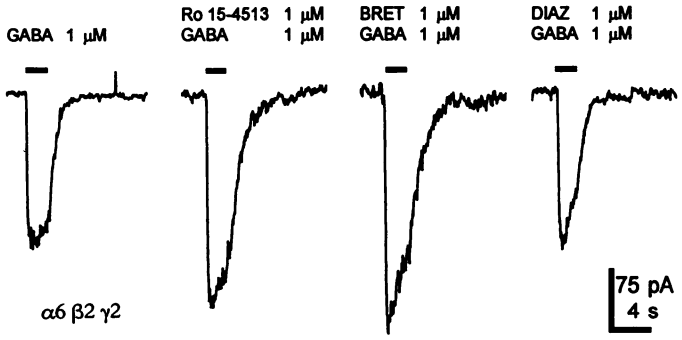

Fig. 5. Effects on the GABA-evoked response of Ro 15–4513, bretazenil, and diazepam sequentially applied to the same cell stably transfected with the cDNAs coding for the $\alpha6\beta2\gamma2$ subunit combination. *First trace on left*, current evoked by a 2-sec control pulse of GABA (1 μM). Ro 15–4513 (1 μM) and bretazenil (*BRET*, 1 μM) potentiated the GABA response (*second and third traces*). Diazepam (*DIAZ*, 1 μM) had no effect on the GABA-evoked current (*fourth trace*). The drugs were preapplied by bath perfusion for ≥1 min before microapplication of the GABA/drug mixture.

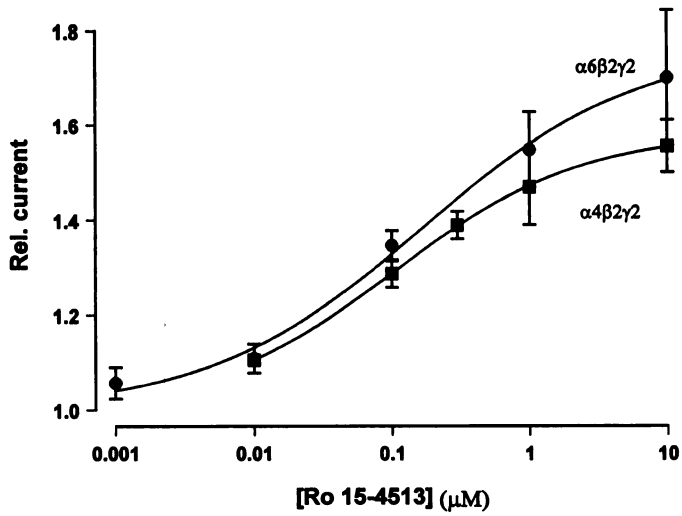

Fig. 6. Dose-response curves for the potentiating effect of Ro 15–4513 on the GABA-evoked current mediated by $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ recombinant $GABA_A$ receptors. The current amplitudes obtained from GABA applications (1 μM) in the presence of different concentrations of Ro 15–4513 are plotted as a function of the drug concentration. The maximum current amplitudes were measured and plotted as described in Materials and Methods. Values were normalized to the control responses obtained with GABA alone. A relative current of 1.0 represents the GABA-induced current in the absence of Ro 15–4513.

maximal levels of potentiation were not significantly different for the two receptor subtypes: 156 ± 6% of control for $\alpha4\beta2\gamma2$ (four experiments) and 170 ± 14% of control for $\alpha6\beta2\gamma2$ (nine experiments) (Fig. 6). To assess any possible dependence of the effect of Ro 15–4513 on the type of transfection (i.e., transient or stable), this drug was also tested on HEK 293 cells transiently transfected with $\alpha6\beta2\gamma2$ subunit cDNAs. At 1 μM, Ro 15–4513 potentiated the GABA-evoked response recorded from these cells (145.0 ± 16.0% of control; four experiments).

Korpi *et al.* (16) reported that furosemide potently blocks the GABA-evoked current mediated by the $\alpha6\beta2\gamma2$ and $\alpha6\beta3\gamma2$ recombinant receptors but not the current mediated by the $\alpha1\beta2\gamma2$ receptor and suggested that furosemide might be the first antagonist selective for these α6 subunit-containing $GABA_A$ receptors. We therefore compared the effect of furosemide on the $\alpha4\beta2\gamma2$ receptor with its action on the $\alpha6\beta2\gamma2$ receptor and found that furosemide blocks the current mediated by the $\alpha4\beta2\gamma2$ receptor with similar dose dependence to its effect on the $\alpha6\beta2\gamma2$-mediated current (Fig. 7). The GABA-evoked response of the $\alpha4\beta2\gamma2$ receptor was decreased by 13.0 ± 6.2% (three experiments) with 10 μM furosemide and 47.9 ± 1.7% (three experiments) with 100 μM. The corresponding values for the $\alpha6\beta2\gamma2$ receptor were 18.4 ± 1.4 (seven experiments) and 60.0 ± 1.9 (six experiments), indicating that the α4- and α6-containing receptors are not readily distinguishable from each another by this channel blocker.

Zn^{2+} blocks the GABA-evoked currents mediated by many recombinant $GABA_A$ receptors lacking the γ2 subunit and consequently insensitive to classic benzodiazepine full agonists (27). Because the recombinant $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ $GABA_A$ receptors are diazepam insensitive, they were tested for Zn^{2+} sensitivity to determine whether Zn^{2+} insensitivity correlates with the presence of a γ2 subunit independent of benzodiazepine full agonist sensitivity. The amplitude of the response evoked by a pulse of 5 μM GABA applied to cells expressing the $\alpha6\beta2\gamma2$ $GABA_A$ receptor was reduced to 46.5 ± 3.0% of the control (seven experiments; Fig. 8A) by 100 μM Zn^{2+}. The blocking action of Zn^{2+} was dose depen-

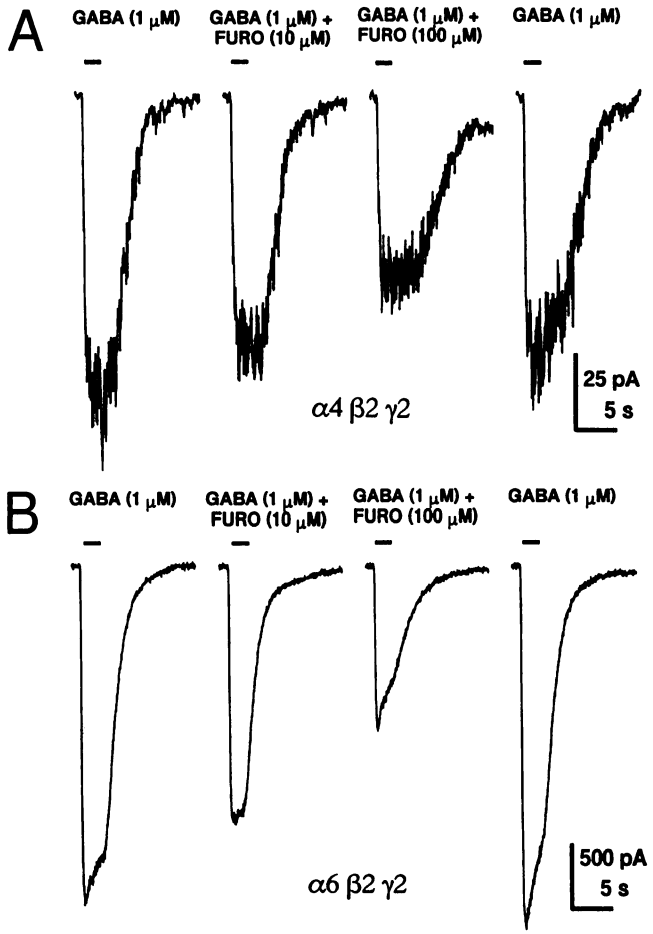

Fig. 7. Effect of furosemide (*FURO*, 10 and 100 μM) on the GABA-evoked responses of (A) the $\alpha4\beta2\gamma2$ and (B) the $\alpha6\beta2\gamma2$ recombinant $GABA_A$ receptors (1 μM GABA). Furosemide reversibly decreased the GABA-induced currents of the two receptors equally at each of the two doses tested.

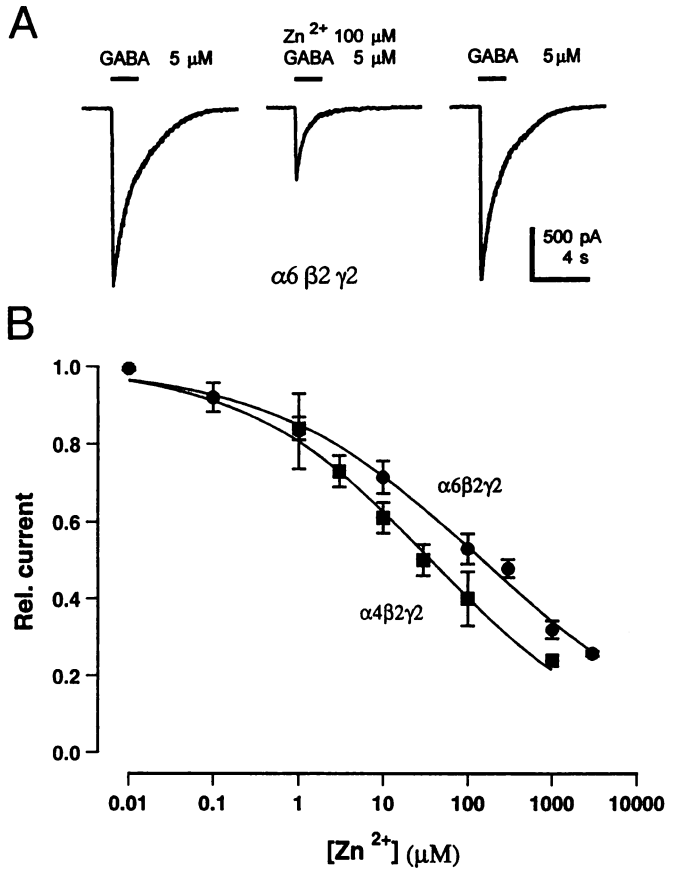

Fig. 8. Effect of Zn^{2+} on the GABA-evoked responses of the $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ recombinant $GABA_A$ receptors. A, Sample traces illustrating the reversible inhibition by Zn^{2+} (100 μM) of the GABA-induced current (5 μM GABA) mediated by the $\alpha6\beta2\gamma2$ $GABA_A$ receptor. B, Dose-response curves of the blocking action of Zn^{2+} on the GABA-evoked currents mediated by the $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ $GABA_A$ receptors. The maximum current amplitudes were measured and plotted as described in Materials and Methods. Values are normalized to the control responses obtained with GABA alone. A relative current of 1.0 represents the GABA-induced current in the absence of Zn^{2+}. Values are mean ± standard error for $\alpha4\beta2\gamma2$ (■, 10 experiments) and $\alpha6\beta2\gamma2$ (●, eight experiments).

dent, with a threshold between 0.01 and 0.1 μM and EC_{50} values of 37.0 ± 4.7 μM ($\alpha4\beta2\gamma2$ receptor, 10 experiments; Fig. 8B) and 149.8 ± 18.9 μM ($\alpha6\beta2\gamma2$ receptor, eight experiments; Fig. 8B). The EC_{50} values were estimated from the fitted curves since the saturating dose of Zn^{2+} could not be determined because prolonged exposure of the cells to the higher concentrations of Zn^{2+} resulted in the loss of the patch [also observed by Kilic *et al.* (28)]. The fitted curves indicated that total block could be expected if loss of the patch did not occur. The difference between the Zn^{2+} sensitivities of the two receptors was small (Fig. 8B).

Modulation of the resting inward current mediated by the $\alpha6\beta2\gamma2$ $GABA_A$ receptor. DMCM, a β-carboline benzodiazepine-binding site ligand and, like Ro 15–4513, an inverse agonist at submicromolar concentrations on α1, α3, and α5 subunit-containing receptors, has also been reported to enhance the GABA-evoked current mediated by $\alpha6\beta2\gamma2$ $GABA_A$ receptors (13). We confirmed this observation and found that 10 μM DMCM evoked a small inward shift of the base-line current and an increase in base-line noise concurrent with the enhancement of the GABA-evoked response (Fig. 9A). These effects were also observed during application of a comparatively high concentration of bretazenil (3 μM; Fig. 9B). This suggested an increase in channel-opening activity. Raising the bretazenil concentration to 10 μM increased the base-line noise and induced a small inward current (Fig. 9B). The most likely explanation for this observation was the presence of a low amplitude, steady state current mediated by the $\alpha6\beta2\gamma2$ receptor and enhanced by the application of bretazenil. To test this hypothesis, picrotoxin and bretazenil were pulse-applied using the microapplicator. The experiments were carried out using a microapplicator that contained no GABA and coverslips of cells that had not been exposed to GABA. After the formation of a gigaseal but before breakthrough to whole-cell recording mode, the recording amplifier was precisely zeroed at high gain and filter cutoff frequency (20 mV/pA, 1 kHz). After breakthrough, a steady state, resting current was evident (Fig. 10, *first trace*). A brief pulse of 20 μM picrotoxin resulted in a deflection of the current trace in the outward direction and a reduction in base-line noise (Fig. 10, *second trace*). A pulse of 10 μM bretazenil evoked a nondesensitizing, inward current and an increase in base-line noise (Fig. 10, *third trace*). Interruption of a bretazenil pulse by a picrotoxin pulse drove the current level back past the resting level to the level

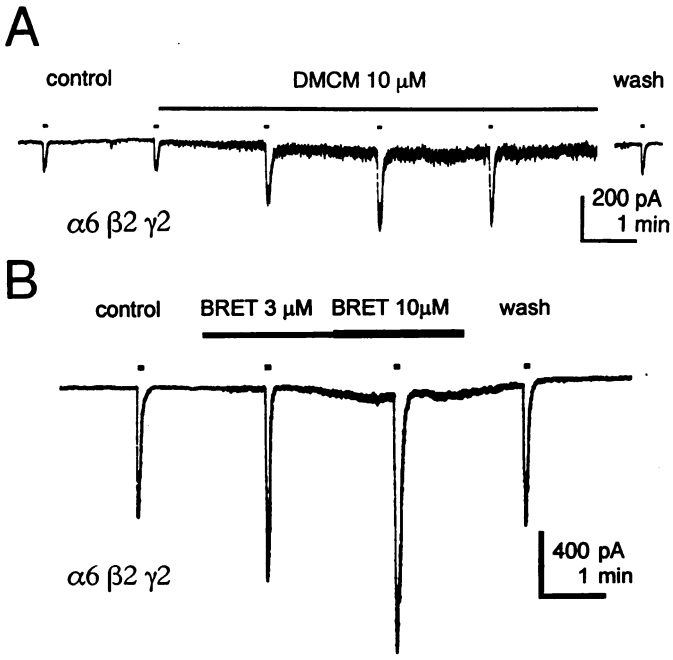

Fig. 9. A, The effect of DMCM on the resting current amplitude and noise level and on the amplitude of the GABA-evoked response of a cell expressing $\alpha6\beta2\gamma2$ receptors. DMCM (10 μM) was applied continuously (*bar*) and resulted in a slow inward shift in the base-line current, an accompanying base-line noise increase, and potentiation of the amplitude of the GABA-evoked current. The postwash response was recorded after 20 min in control saline. *Short bars*, 2-sec applications of GABA (1 μM) and DMCM. B, The effect of bretazenil (*BRET*) on the resting current amplitude and noise level and on the amplitude of the GABA-evoked response of a cell expressing $\alpha6\beta2\gamma2$ receptors. The cell was exposed to bretazenil alone (3 μM; *thin bar*), resulting in an increase in base-line noise and a potentiated GABA-evoked current. An increase in the bretazenil concentration to 10 μM (*thick bar*) further enhanced the GABA-evoked current, further increased the base-line noise, and induced a slow inward shift in the base-line current. When bretazenil was removed from the bath solution, the base-line and GABA-evoked responses reverted to the control values.

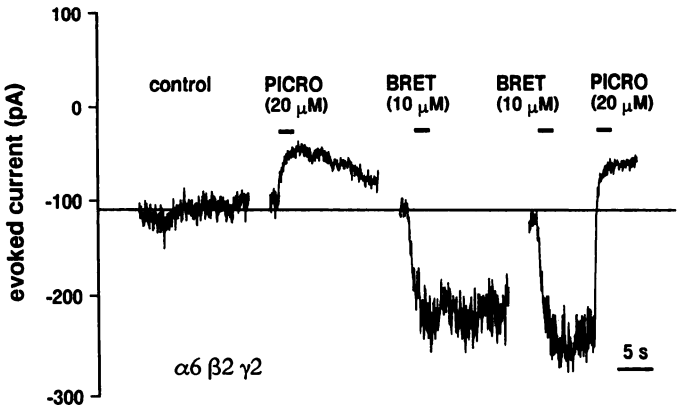

Fig. 10. Effect of picrotoxin on the resting and bretazenil-evoked current levels. *Ordinate*, absolute membrane current, revealing a resting, inward base-line current of ~100 pA. Picrotoxin (*PICRO*, 20 μM) induced a reversible outward shift in the base-line current accompanied by a reduction in base-line noise level. Bretazenil (*BRET*, 10 μM) induced a nondesensitizing, reversible inward current and an increase in base-line noise level. The application of a pulse of picrotoxin during the course of the bretazenil-evoked current caused the membrane current to shift to the absolute level induced by picrotoxin alone, indicating that both the resting current level and the bretazenil-evoked current are blocked by picrotoxin.

of outward current reached during application of picrotoxin in isolation (Fig. 10, *fourth trace*). These results confirm that the $\alpha6\beta2\gamma2$ $GABA_A$ receptor conducts a low level of steady state current in the absence of GABA and that this current can be potentiated by bretazenil and blocked by picrotoxin in the same way as the GABA-evoked current. This steady state resting current was observed in all eight $\alpha6\beta2\gamma2$ receptor-expressing cells tested for this phenomenon. A similar explanation most likely accounts for the effect of DMCM on the base-line current amplitude and noise level that occur in addition to its potentiating effect on the amplitude of the GABA-evoked current mediated by $\alpha6\beta2\gamma2$ receptors (Fig. 9A). Both 10 μM flumazenil and 10 μM Ro 15–4513 evoked currents similar to but smaller than those evoked by bretazenil (data not shown). It was thus not possible to apply a sufficiently high dose of flumazenil to determine whether it could block the direct action of bretazenil. Neither an increase in base-line noise level nor the direct activation of an inward current was induced by bretazenil, Ro 15–4513, or flumazenil in any of the six cells expressing $\alpha4\beta2\gamma2$ $GABA_A$ receptors tested, and no clear effect of picrotoxin by itself on the resting current amplitude or noise level could be discerned. These observations suggest that spontaneous $GABA_A$ channel activation in the absence of GABA is restricted to the $\alpha6\beta2\gamma2$ receptor or occurs to a substantially lower degree in the $\alpha4\beta2\gamma2$ receptor.

Analysis of $\alpha4\beta2$ and $\alpha4\gamma2$ receptors. In membranes of HEK 293 cells transiently transfected with the cDNAs coding for α4, β2, and γ2 subunits or permanently transfected with the cDNAs coding for α6, β2, and γ2 subunits, all of the corresponding subunit proteins were detected by Western blotting. It is therefore assumed that the expressed $GABA_A$ receptors were wholly or predominantly the $\alpha4\beta2\gamma2$ or the $\alpha6\beta2\gamma2$ receptors. To test whether the potential formation of double-subunit receptors, in addition to the triple-subunit receptors, might contribute to the pharmacological properties of the drugs tested, HEK 293 cells were transfected with the cDNAs encoding $\alpha4\beta2$ or $\alpha4\gamma2$ subunit combinations. Functional receptors were found that responded to pulses of GABA with currents of ≤200 pA (1 sec, 1 μM GABA) and ≤150 pA (1 sec, 10 μM GABA), respectively. The response of the $\alpha4\beta2$ receptor to 1 μM GABA was insensitive to flunitrazepam, bretazenil, and Ro 15–4513 (1 μM each), but the response of the $\alpha4\gamma2$ receptor to 1 μM GABA, also insensitive to flunitrazepam (1 μM), was potentiated by bretazenil and Ro 15–4513 (1 μM each) in a similar manner to the $\alpha4\beta2\gamma2$ receptor-mediated response. Thus, if there is appreciable expression of $\alpha4\gamma2$ receptors, it cannot be excluded that these receptors contribute to the pharmacological effects observed.

Discussion

In brain, the α4 and α6 subunit-containing $GABA_A$ receptors exhibit different distribution patterns (2, 3, 29), suggesting that they might have special functions in signal transduction and drug modulation. The properties of the recombinant $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors were therefore investigated. The two receptors displayed the highest GABA sensitivities reported for $\alpha x\beta2\gamma2$ receptors, with GABA EC_{50} values of 3.9 and 1.3 μM, respectively. The Hill coefficients suggest almost no cooperativity in gating the channel. Although the $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors were known to be insensitive to the classic benzodiazepine full agonist diazepam, we found that they are nevertheless modulated by some partial and inverse benzodiazepine agonists. However, not only the partial agonist but also the inverse agonists that we tested potentiated the GABA-evoked response of the $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors. The EC_{50} values for the respective modulators were similar for the two receptors. In addition, the two receptors were almost equally sensitive to blockade by furosemide and Zn^{2+}. The major difference between them was a 2-fold difference in the maximal potentiation of the GABA-evoked response by the partial agonist bretazenil (Fig. 3).

It was known from radioligand displacement binding data that [^{3}H]Ro 15–4513 bound with high affinity to the $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors (2, 3, 30), the latter result being in line with binding data from diazepam-insensitive sites in the cerebellum (15). The ligand-binding spectrum has been extended. Bretazenil, a partial agonist at the benzodiazepine-binding site, had a K_i value (20 nM) only 3-fold higher than that of Ro 15–4513 (K_D = 7 nM), a result recently corroborated by Yang *et al.* (30), who reported that the human α4 subunit expressed in combination with β2 and γ2(long) subunits forms a diazepam-insensitive receptor that binds the partial agonist bretazenil in addition to Ro 15–4513 and DMCM. Flumazenil and DMCM had intermediate affinities. The binding profiles of the compounds revealed little difference in affinity between the two receptors.

Bretazenil has been shown in numerous studies *in vivo* and on recombinant receptors to be a partial agonist acting at the classic benzodiazepine-binding site of $GABA_A$ receptors (24, 31). We report that bretazenil also potentiates the GABA response at $\alpha4\beta2\gamma2$ and $\alpha6\beta2\gamma2$ receptors (Fig. 3), which are completely insensitive to diazepam. Thus, modulation of neuronal circuits incorporating α4 and α6 subunit-containing receptors might contribute to the pharmacological profile of bretazenil. Such actions might be linked to an increased risk of motor impairment, as shown for a mutant α6 receptor (32). Not all partial agonists with a benzodiazepine structure in-

teract with α4 and α6 subunit-containing receptors; imidazenil did not displace [^{3}H]Ro 15–4513 from the α4β2γ2 and α6β2γ2 receptors in the binding assay (Table 1) and was inactive when tested electrophysiologically on the α6β2γ2 receptor.

Ro 15–4513, an azido analog of the benzodiazepine antagonist flumazenil (Ro 15–1788), was originally characterized as a partial inverse agonist of the benzodiazepine-binding site in the central nervous system on the basis of behavioral, binding, and electrophysiological data (33) and of its stimulatory effects on substantia nigra pars reticulata neurons (34). However, we found that Ro 15–4513 enhanced the GABA-evoked response mediated by recombinant α4β2γ2 and α6β2γ2 receptors over a concentration range of 0.01–10 μM. In contrast to our data, Kleingoor *et al.* (14) reported that the GABA response mediated by α6β2γ2 receptors transiently expressed in 293 cells was decreased (−28.5%) by 1 μM Ro 15–4513. This discrepancy between their results and our data is unresolved; it is not the result of the transfection procedure because both transiently and permanently transfected cells yielded the same potentiating response.

Flumazenil is an antagonist at the benzodiazepine full agonist binding site; when applied at 1 μM, it partially blocked the potentiating effect of 1 μM bretazenil on the α6β2γ2 $GABA_A$ receptor (Fig. 4). Partial rather than full block might be a result of an affinity for the [^{3}H]Ro 15–4513-binding site of the α6β2γ2 receptor that is 10-fold less than that of bretazenil (Table 1). When applied at 10 μM to the cells expressing the α6β2γ2 receptor, flumazenil by itself weakly potentiated the GABA-evoked response.

The β-carboline inverse agonist DMCM, when tested at 10 μM, potentiated the GABA-evoked current mediated by the α6β2γ2 receptor (Fig. 9A). It should, however, be noted that DMCM has a biphasic dose-response curve on the α1β2γ2 receptor, reducing the GABA response at concentrations of ≤1 μM but potentiating it at higher concentrations (13, 35). Bretazenil and DMCM (Fig. 9, A and B), as well as Ro 15–4513 and flumazenil, increased the base-line noise and evoked a small, nondesensitizing inward current in cells expressing the α6β2γ2 receptor. The blocking action of pulse-applied picrotoxin indicated that these effects represent a potentiation of spontaneous, steady state α6β2γ2 receptor channel activity that occurs in the absence of GABA. This phenomenon could not be detected in cells expressing α4β2γ2 receptors, either because the resting channel activity was too low or because it did not occur. This seems to be the first report of spontaneous activity in a recombinant $GABA_A$ receptor consisting of three different subunit subtypes. Apparently, direct activation by 100 nM 3α-hydroxy-5α-pregnan-20-one, a neuroactive steroid, of α6β2γ2 $GABA_A$ receptors in the absence of GABA (11) might also be due to the potentiation by the steroid of the spontaneous, steady state channel activity. Homomeric $GABA_A$ receptors consisting of bovine α subunits or human, rat, or molluscan β subunits have been reported to be spontaneously active in the absence of GABA and to be blocked by picrotoxin (36–39), and Khrestchatisky *et al.* (40) made similar observations for a hetero-oligomeric $GABA_A$ receptor composed of the α5 and a β subunit. Spontaneously active GABA-sensitive channels have been observed in intermediate lobe cells from the pituitary (41), indicating that *in vivo* this phenomenon may not be confined to α6-containing receptors, and we observed similar effects in α2β3γ2 recombinant receptors.[3]

Furosemide and Zn^{2+}, two agents that selectively reduce the GABA-evoked responses mediated by $GABA_A$ receptors of certain subunit combinations (16, 27), had quantitatively similar effects on the α4β2γ2 and α6β2γ2 receptors. Furosemide, which is reported to be comparatively inactive on α1β2γ2 receptors (16), blocked the α4β2γ2 and α6β2γ2 receptor-mediated current by 60% and 48%, respectively, at 100 μM (Fig. 7). Its selectivity thus includes not only an α6 subunit-containing receptor (16) but also a receptor containing the α4 subunit.

Diazepam-insensitive $GABA_A$ receptors are thought to be more sensitive to Zn^{2+} than diazepam-sensitive receptors (42). In addition, the absence of a γ subunit in a recombinant receptor, which eliminates diazepam sensitivity, generally increases the Zn^{2+} sensitivity of the receptor, with the majority of the sensitive combinations being almost totally blocked by 5–10 μM Zn^{2+}. GABA currents mediated by subunit combinations containing a γ2 subunit are only slightly reduced by 10 and 100 μM Zn^{2+} (27, 42). However, the GABA-evoked currents mediated by the α4β2γ2 and α6β2γ2 receptors were more sensitive to Zn^{2+} than other γ2 subunit-containing recombinant receptors reported previously (43). The EC_{50} value for the α6β2γ2 receptor was 149.8 ± 18.9 μM, which is in line with the Zn^{2+} sensitivity of the GABA-evoked responses of cerebellar granule cells in culture (EC_{50} = 57.2 μM (28)). Possibly, diazepam insensitivity *per se*, rather than the absence of a γ2 subunit, correlates with Zn^{2+} sensitivity.

A likely Zn^{2+}-binding site was proposed to exist on the extracellular flank of the M2 membrane spanning domain (43). In particular, position 285 on the γ2 subunit is occupied by a positively charged residue (lysine), whereas the homologous region on the α and β subunits has a net negative charge (aspartate and glutamate), which was considered suitable for a possible Zn^{2+}-binding site in receptors lacking a γ2 subunit (43). However, the homologous residue on the α6 subunit is a histidine (2), which would predict because of its positive charge an even weaker Zn^{2+} binding to the α6β2γ2 receptors. However, a higher Zn^{2+} sensitivity was found for the α6β2γ2 receptor than for the α1β2γ2 receptor. The residues homologous to the γ2 lysine at position 285 are thus unlikely to play a predominant part in Zn^{2+} binding.

In conclusion, α4 and α6 subunit-containing $GABA_A$ receptors display reversed efficacy for some ligands of the classic benzodiazepine-binding site (e.g., Ro 15–4513, DMCM). In addition, they are pharmacologically restricted with regard to benzodiazepine-induced potentiation. Although insensitive to classic full agonists such as diazepam, they contribute to the pharmacological profile of the partial agonist bretazenil, which potentiates both α4 and α6 subunit-containing receptors. *In vivo*, benzodiazepine-induced motor impairment is correlated with mutant, diazepam-sensitive α6 subunit-containing receptors (32). This suggests that potentiation of α6 subunit-containing receptors may be unfavorable to the pharmacological profile of a drug.

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