Pharmacological Properties of GABA$_A$ Receptors Containing γ1 Subunits$^{\text{S}}$

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ABSTRACT

GABA$_A$ receptors composed of α_1, β_2, γ_1 subunits are expressed in only a few areas of the brain and thus represent interesting drug targets. The pharmacological properties of this receptor subtype, however, are largely unknown. In the present study, we expressed $\alpha_1\beta_2\gamma_1$-GABA$_A$ receptors in *Xenopus laevis* oocytes and analyzed their modulation by 21 ligands from 12 structural classes making use of the two-microelectrode voltage-clamp method and a fast perfusion system. Modulation of GABA-induced chloride currents (I_{GABA}) was studied at GABA concentrations eliciting 5 to 10% of the maximal response. Triazolam, clotiazepam, midazolam, 2-(4-methoxyphenyl)-2,3,5,6,7,8,9,10-octahydro-cyclohepta-(*b*)pyrazolo[4,3-*d*]pyridin-3-one (CGS 20625), 2-(4-chlorophenyl)-pyrazolo[4,3-*c*]quinolin-3-one (CGS 9896), diazepam, zolpidem, and bretazenil at 1 μM concentrations were able to significantly (>20%) enhance I_{GABA} in $\alpha_1\beta_2\gamma_1$ receptors. Methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, 3-methyl-6-[3-trifluoromethyl-phenyl]-1,2,4-triazolo[4,3-*b*]pyridazine (Cl 218,872), clobazam, flumazenil, 5-(6-ethyl-7-methoxy-5-methylimidazo[1,2-*a*]pyrimidin-2-yl)-3-methyl-[1,2,4]-oxadiazole (Ru 33203), 2-phenyl-4-(3-ethyl-piperidinyl)-quinoline (PK 9084), flurazepam, ethyl-7-methoxy-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*]pyrrolo[2,1-*c*] [1,4]benzodiazepine-1-carboxylate (L-655,708), 2-(6-ethyl-7-methoxy-5-methylimidazo[1,2-*a*]pyrimidin-2-yl)-4-methyl-thiazole (Ru 33356), and 6-ethyl-7-methoxy-5-methylimidazo[1,2-*a*]pyrimidin-2-yl)phenylmethanone (Ru 32698) (1 μM each) had no significant effect, and flunitrazepam and 2-phenyl-4-(4-ethyl-piperidinyl)-quinoline (PK 8165) inhibited I_{GABA}. The most potent compounds triazolam, clotiazepam, midazolam, and CGS 20625 were investigated in more detail on $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2\gamma_{2S}$ receptors. The potency and efficiency of these compounds for modulating I_{GABA} was smaller for $\alpha_1\beta_2\gamma_1$ than for $\alpha_1\beta_2\gamma_{2S}$ receptors, and their effects on $\alpha_1\beta_2\gamma_1$ could not be blocked by flumazenil. CGS 20625 displayed the highest efficiency by enhancing at 100 μM I_{GABA} ($\alpha_1\beta_2\gamma_2$) by 775 $\pm$ 17% versus 526 $\pm$ 14% I_{GABA} ($\alpha_1\beta_2\gamma_1$) and 157 $\pm$ 17% I_{GABA} ($\alpha_1\beta_2$) ($p < 0.05$). These data provide new insight into the pharmacological properties of GABA$_A$ receptors containing γ1 subunits and may aid in the design of specific ligands for this receptor subtype.

GABA is the principal inhibitory neurotransmitter in the mammalian brain. It mediates fast synaptic inhibition by interaction with the GABA$_A$ receptor. GABA$_A$ receptors are ligand-gated ion channels that are modulated by a large number of clinically relevant drugs such as benzodiazepines (BZs), barbiturates, neurosteroids, and anesthetics (Sieghart, 1995). They are assembled from individual subunits forming a pentameric structure. Nineteen isoforms of mammalian GABA$_A$ receptor subunits have been cloned: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ϵ, p, ρ_{1-3}, and θ (Barnard et al., 1998; Simon et al., 2004). The major receptor subtype of the GABA$_A$ receptor in adults consists of α_1, β_2, and γ_2 subunits, and the most likely stoichiometry is two α subunits, two β subunits, and one γ subunit (Sieghart and Sperk, 2002).

ABBREVIATIONS: CGS 20625, 2-(4-methoxyphenyl)-2,3,5,6,7,8,9,10-octahydro-cyclohepta-(*b*)pyrazolo[4,3-*d*]pyridin-3-one; CGS 9896, 2-(4-chlorophenyl)-pyrazolo[4,3-*c*]quinolin-3-one; I_{GABA}, GABA-induced chloride currents; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; Cl 218,872, 3-methyl-6-[3-trifluoromethyl-phenyl]-1,2,4-triazolo[4,3-*b*]pyridazine; Ru 33203, 5-(6-ethyl-7-methoxy-5-methylimidazo[1,2-*a*]pyrimidin-2-yl)-3-methyl-[1,2,4]-oxadiazole; PK 9084, 2-phenyl-4-(3-ethyl-piperidinyl)-quinoline; L-655,708, ethyl-7-methoxy-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*]pyrrolo[2,1-*c*] [1,4]benzodiazepine-3-carboxylate; Ru 33356, 2-(6-ethyl-7-methoxy-5-methylimidazo[1,2-*a*]pyrimidin-2-yl)-4-methyl-thiazole; Ru 32698, 6-ethyl-7-methoxy-5-methylimidazo[1,2-*a*]pyrimidin-2-yl)phenylmethanone; PK 8165, 2-phenyl-4-(4-ethyl-piperidinyl)-quinoline; Ro 15-1788, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate; Ru 31719, (7-ethyl-5-methoxyimidazo[1,2-*a*]quinolin-2-yl)phenyl methanone; BZ, benzodiazepine; Ro 15-1788, flumazenil; Ro 15-4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo-1,4-benzodiazepine-3-carboxylate; β-CCM, methyl-β-carboline-3-carboxylate; ANOVA, analysis of variance.

The subunit composition determines the GABA sensitivity and the pharmacological properties of the GABA$_A$ receptor (Sieghart, 1995; Hevers and Luddens, 1998; Boileau et al., 2002). The subunit composition of the receptor also affects the time course of the GABA response (desensitization and deactivation of the chloride currents) (Bianchi et al., 2001; Boileau et al., 2003; Feng et al., 2004). Mutation of amino acid residues in α and γ_2 subunits modulate the BZ sensitivity of the receptor, suggesting that the BZ binding pocket is located at the interface between α and γ_2 (Sigel, 2002; Ernst et al., 2003). There is clear evidence that substitution of the γ_2 subunit by either γ_1 or γ_3 significantly alters the sensitivity for BZ (Hevers and Luddens, 1998).

In contrast to the γ_2 subunit, which is ubiquitously expressed in the central nervous system, the γ_1 subunit is expressed in only a few areas of the brain such as the amygdala (central and medial nuclei), the pallidum, the septum, the substantia nigra, and the thalamus (centrolateral and medial nuclei) (Pirker et al., 2000; Korpi et al., 2002). Compounds selectively interacting with receptors containing γ_1 subunits thus might have a substantial clinical potential.

Compared with receptors containing γ_2 subunits, little is known about the pharmacological profile of GABA$_A$ channels composed of α_1, β_2, and γ_1 subunits. Ymer et al. (1990) observed a loss in affinity for the benzodiazepine antagonist Ro 15-1788 and the inverse agonist methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) when the γ_1 was substituted for γ_2 in $\alpha_1\beta_1\gamma_2$ receptors. Negative modulatory effects of Ro 15-4513, β-CCM, and DMCM for GABA$_A$ receptors composed of $\alpha_{1/2/3}\beta_1\gamma_2$ subunits are changed to positive modulatory effects in $\alpha_{1/2/3}\beta_1\gamma_1$ receptors (Puia et al., 1991; Wafford et al., 1993). Benke et al. (1996) observed a low affinity for clonazepam, zolpidem, and flunitrazepam and apparent insensitivity for flumazenil and Ro 15-4513 for γ_1-containing receptors. Wafford et al. (1993) demonstrated a reduced enhancement of chloride currents through $\alpha_3\beta_2\gamma_3$ by diazepam, clonazepam, and bretazenil compared with $\alpha_3\beta_2\gamma_2$ and a negative modulatory effect of zolpidem for $\alpha_2\beta_1\gamma_1$ and alpidem for $\alpha_3\beta_1\gamma_1$ receptors.

Overall, in 4 different studies, a total of 14 compounds from 5 different compound classes have been investigated so far for their ability to modulate GABA$_A$ receptors containing γ_1 subunits. Unfortunately, most of these studies were carried out under different experimental conditions and with receptors containing different α and β subunits combined with γ_1. Thus, the relative efficacies of these compounds for $\alpha\beta\gamma_1$ are not comparable (Hevers and Luddens, 1998).

In the present study, we analyzed the modulation of $\alpha_1\beta_2\gamma_1$ receptors expressed in *Xenopus laevis* oocytes by 21 compounds comprising distinct chemical structures. Triazolam, clotiazepam, midazolam, and CGS 20625 exhibited a significant potency and efficiency, whereas the other compounds were either inactive or displayed only a low potency on γ_1-containing receptors.

Materials and Methods

Chemicals. Compounds were obtained from the following sources: flunitrazepam (7-nitro-1,3-dihydro-1-methyl-5-*o*-fluorophenyl-2*H*-1,4-benzodiazepin-2-one), diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one), flurazepam (7-chloro-1,3-dihydro-1-ethylaminodiethyl-5-*o*-fluorophenyl-2*H*-1,4-benzodiazepin-2-one),

midazolam [8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine], Ro 15-1788, and bretazenil [*t*-butyl(*s*)-8-bromo-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*]pyrrolo[2,1-*c*] [1,4]benodiazepine-1-carboxylate] were from Hoffmann La Roche (Basel, Switzerland); L-655,708 was purchased from Tocris Cookson Inc. (Bristol, UK); clotiazepam [5-(2-chlorophenyl)-7-ethyl-1,3-dihydro-1-methyl-2*H*-thieno[2,3-*e*][1,4]diazepin-2-one] was from Troponwerke (Köln, Germany); clobazam [7-chloro-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4(3*H*,5*H*)-dione] was from sanofi-aventis (Bridgewater, NJ); triazolam [8-chloro-6-(2-chlorophenyl)-1-methyl-4*H*-1,2,4-triazolo[4,3-*a*][1,4]benzodiazepine] was from Sigma (Vienna, Austria); DMCM was from Ferrosan (Soeborg, Denmark); CGS 9896 and CGS 20625 were from Novartis (Basel, Switzerland); zolpidem [*N*,*N*,6-trimethyl-2-(4-methylphenyl)imidazo[1,2-*a*]-pyridine-3-acetamide] was from Synthelabo Recherche (Bagneux, France); Cl 218,872 was from American Cyanamide Comp. (Wayne, NJ); Ru 31719, Ru 32698, Ru 33203, and Ru 33356 were from Roussel Uclaf (Romainville, France); PK 8165 and PK 9084 were from Pharmuka Laboratories (Gennevilliers, France). For chemical structures, see Ogris et al. (2004).

Expression and Functional Characterization of GABA$_A$ Receptors. *X. laevis* oocytes were prepared and injected as described previously (Grabner et al., 1996). Female *X. laevis* (Nasco, Fort Atkinson, WI) were anesthetized by exposing them for 15 min to a 0.2% MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester; Novartis) solution before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase (type 1A, Sigma). One day after isolation, the oocytes were injected with approximately 10 to 50 nl of a solution of diethyl pyrocarbonate water containing the different cRNAs at a concentration of approximately 300 to 3000 pg/nl/subunit. The amount of cRNA was determined by means of a NanoDrop ND-1000 (Kisker-Biotech, Steinfurt, Germany). To ensure expression of the γ subunit in the case of $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2\gamma_{2S}$ receptors, cRNAs were mixed in a ratio of 1:1:10, and for receptors comprising only α_1 and β_2 subunits, they were mixed in a ratio of 1:1 (Boileau et al., 2002).

Oocytes were stored at 18°C in ND96 solution (Methfessel et al., 1986). Electrophysiological experiments were performed by the two-electrode voltage-clamp method making use of a TURBO TEC 01C amplifier (NPI Electronic GmbH, Tamm, Germany) at a holding potential of -70 mV. The bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 5 mM HEPES, pH 7.4.

Perfusion System. GABA was applied by means of a modified version of a fast perfusion system according to Hering (1998). A schematic drawing of the perfusion chamber and drug application device is shown in Fig. 1A. As described previously, the voltage-clamp experiments on *X. laevis* oocytes were performed in a small ($\approx$15 μl) bath that was covered by a glass plate. Two angular inlet channels in the glass cover (diameter, <1 mm) enabled access of the two microelectrodes to the oocyte. A funnel for drug application surrounded both access channels for the microelectrodes compared with a funnel surrounding a single access channel in Hering (1998). This modification increased the stability of oocyte perfusion.

Drug or control solutions were applied to the funnel by means of a Miniprep 60 (Tecan, Durham, NC) that was controlled by a DigiData 1322A (Clampex version 9.2; Molecular Devices, Sunnyvale, CA), permitting automation of the experiments [see also Supplemental Videos S1 and S2 (files methods_1.avi and methods_2.avi) for animation of the solution exchange].

To estimate the rate of solution exchange independent of the ligand-receptor interaction, we expressed K$_v$1.1 channels in *X. laevis* oocytes and analyzed the time course of current decay during a rapid increase of the extracellular potassium concentration from 1 to 10 mM (sodium was reduced to 80 mM, respectively). Figure 1B illustrates a typical K$_v$1.1 current during a voltage-clamp step from -80 to $+20$ mV. The current decrease upon fast perfusion with 10 mM potassium at a speed of 1 ml/s is shown on the right in Fig. 1B. A

mean current decline time $t_{10-90\%}$ of 140.8 ± 17.5 ms ($n = 7$) was estimated.

To elicit GABA-induced chloride currents (I_{GABA}), the chamber was perfused with 120 μl of GABA-containing solution at the same volume rate (1 ml/s). The rise time of I_{GABA} ranged usually between 100 and 250 ms (Fig. 1C), which is comparable with the rate of solution exchange estimated in Fig. 1B.

After the initial fast perfusion step for rapid agonist application, the chamber was continuously perfused at a rate of 1 μl/s for a total of 18 s. Before rapid washout of agonist and/or drug, the funnel was emptied by a suction pulse applied to the two funnel outlets (Fig. 1A).
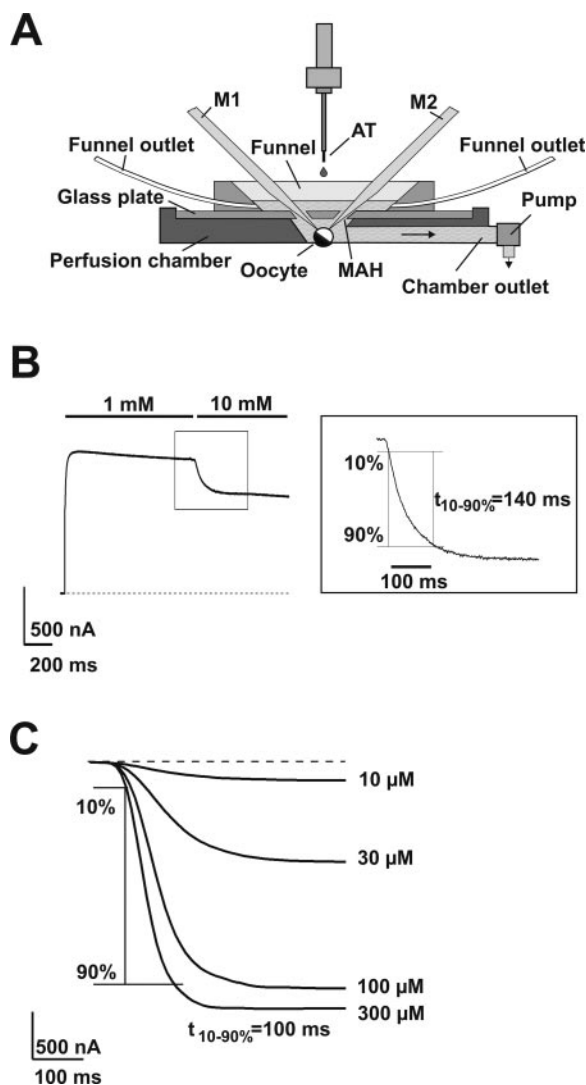
The time between this suction pulse and the application of new solution to the funnel was <1 s to avoid evaporation of the bath surrounding the oocyte.

Duration of washout periods was extended from 3 to 30 min, with increasing concentrations of applied GABA to account for slow recovery from increasing levels of apparent desensitization. Oocytes with maximal current amplitudes >3 μA were discarded to minimize voltage-clamp errors.

Analyzing Concentration-Response Curves. Enhancement of chloride currents by modulators of the GABA$_A$ receptor was measured at a GABA concentration eliciting between 5 and 10% of the maximal current amplitude (EC$_{5-10}$). The EC$_{5-10}$ (usually ranging between 3 and 8 μM) was determined at the beginning of each experiment.

Enhancement of the chloride current (I_{GABA}) was defined as ($I_{(GABA + Comp)}/I_{GABA}) - 1$, where $I_{(GABA + Comp)}$ is the current response in the presence of a given compound, and I_{GABA} is the control GABA current. To measure the sensitivity of the GABA$_A$ receptor for a
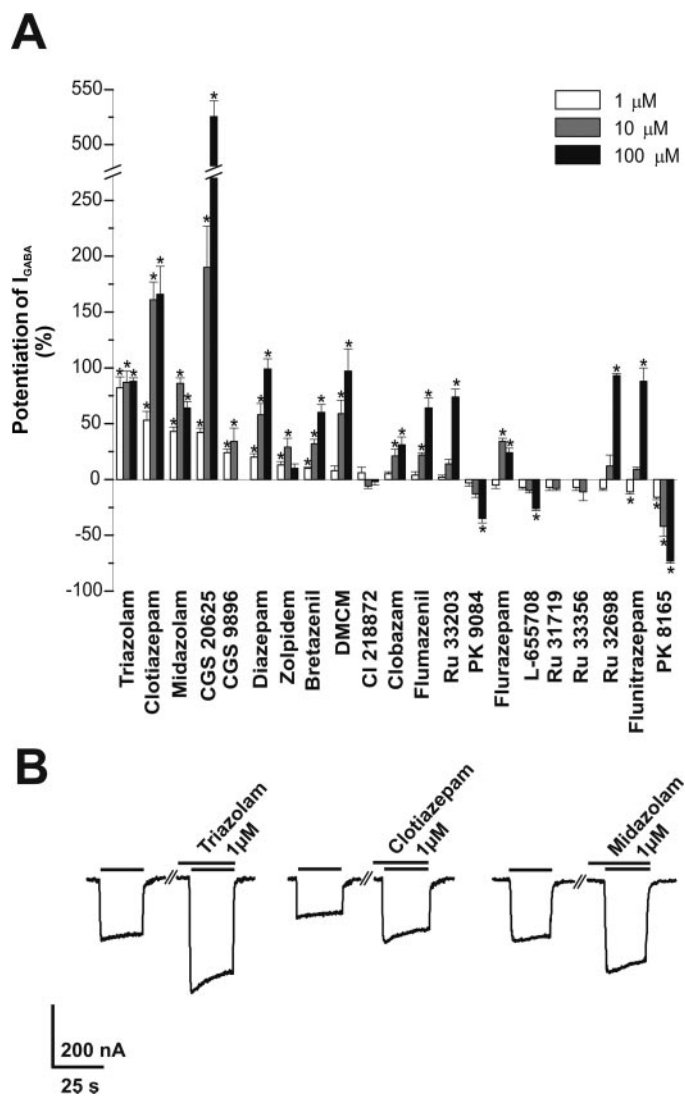

Fig. 1. A, cross-section view of the oocyte perfusion chamber. Two microelectrodes (M1 and M2) are inserted into the angular access inlets in a glass plate (the inlets serve simultaneously as perfusion holes) covering the small (≈15 μl) oocyte chamber. Drug was applied by a Tecan Miniprep 60 application tube (AT) to a quartz funnel surrounding the microelectrode access holes (MAH). Residual solution was removed from the funnel before drug application via the funnel outlets (see Hering, 1998 and the two Supplemental Video files S1 and S2). B, estimation of the rate of solution exchange on an oocyte expressing Kv 1.1 the extracellular potassium concentration was rapidly increased from 1 to 10 mM during a potassium outward current (voltage step from −80 to +20 mV). The time of current decrease (right) from 10 to 90% ($t_{10-90\%}$) was taken as a measure of the volume rate of oocyte perfusion. C, typical time courses of I_{GABA} ($\alpha_1\beta_2\gamma_{2S}$ receptors) activated by different GABA concentrations. Upon application of 300 μM GABA, I_{GABA} increased from 10 to 90% within 100 ms. At 100, 30, and 10 μM GABA $t_{10-90\%}$ was 140, 180, and 230 ms, respectively.

Fig. 2. A, modulation of chloride currents through GABA$_A$ receptors composed of $\alpha_1\beta_2\gamma_1$ subunits by 1 μM (□), 10 μM (▦), and 100 μM (■) of the indicated compounds. Each value represents the mean ± S.E. from at least four oocytes and ≥2 oocyte batches. ∗, significantly different from 0 ($p < 0.05$, t test by ANOVA). B, typical traces for enhancement of chloride currents through $\alpha_1\beta_2\gamma_1$ channels by triazolam, clotiazepam, and midazolam at EC$_{5-10}$. Control currents (GABA, single bar) and corresponding currents elicited by coapplication of GABA and the indicated compound (double bar) are shown.

given compound, it was applied for an equilibration period of 1 min before concomitant application of GABA (EC$_{5-10}$) and increasing concentrations of the compound. None of the compounds investigated was able to induce chloride flux in the absence of GABA. Concentration-response curves were generated, and the data were fitted by nonlinear regression analysis using ORIGIN software (OriginLab Corp, Northampton, MA). Data were fitted to the equation $1/(1 + (EC_{50}/[Comp])^{n_H})$, where EC$_{50}$ is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50%, and n_H is the Hill coefficient. Data are given as mean $\pm$ S.E. from at least four oocytes and ≥ 2 oocyte batches. Statistical significance was calculated using unpaired Student's t test with a confidence interval of $p < 0.05$.

Results

Modulation of $\alpha_1\beta_2\gamma_1$ Receptors. The aim of the present study was to investigate the pharmacological properties of compounds interacting with $\alpha_1\beta_2\gamma_1$ GABA$_A$ receptors. We have therefore analyzed the modulation of this GABA$_A$ receptor subtype by 21 compounds from 12 different structural classes comprising 1,4-benzodiazepines (flunitrazepam, diazepam, flurazepam, midazolam, and triazolam), 1,4-thienodiazepines (clotiazepam), 1,5-benzodiazepines (clobazam), imidazobenzodiazepines (flumazenil, bretazenil, and L-655,708), β-carbolines (DMCM), pyrazoloquinolines (CGS 9896), pyrazolopyridines (CGS 20625), imidazopyridines (zolpidem), triazolopyridazines (Cl 218,872), imidazoquinolines (Ru 31719), imidazopyrimidines (Ru 32698, Ru 33203, and Ru 33356), and quinolines (PK 8165 and PK 9084).

In a first step, GABA$_A$ receptors were activated by GABA concentrations corresponding to EC$_{5-10}$, and drug effects were screened at a single concentration of 1 μM. As illustrated in Fig. 2 ($\square$), only the benzodiazepines triazolam, midazolam, and diazepam, the thienodiazepine clotiazepam, the pyrazolopyridine CGS 20625, and the pyrazoloquinoline

CGS 9896 induced an enhancement of >20% at this concentration. The other compounds induced either a very small but statistically significant enhancement (zolpidem and bretazenil), no statistically significant effect (DMCM, Cl 218,872, clobazam, flumazenil, Ru 33203, PK 9084, flurazepam, L-655,708, Ru 31719, Ru 33356, and Ru 32698), or even an inhibition of GABA-induced chloride currents (flunitrazepam and PK 8165), and details are given in Table 1.

To establish compounds displaying low potency but high efficiency, all compounds were subsequently tested at higher concentrations (10 and 100 μM) (Fig. 2A). Modulation of GABA receptors at low GABA concentrations (EC$_{5-10}$ close to "tonic concentrations") can substantially differ from modulation at high concentrations (i.e., millimolar "synaptic concentrations"). We have, therefore, analyzed the effects of all 21 compounds at 1 mM GABA (Fig. 3A). None of the compounds, however, substantially enhanced or inhibited I_{GABA}. Representative chloride currents induced by 1 mM in the absence or presence of 1 μM triazolam or CGS 20625 are shown in Fig. 3B.

Contribution of $\alpha_1\beta_2$ Receptors. Previous studies have clearly shown that the extent of the incorporation of γ subunits into heterologously expressed GABA$_A$ receptors may vary between oocyte batches and decrease with time (Boileau

TABLE 1
Modulation of the GABA-induced chloride currents by 1 μM concentration of the indicated compounds for receptors composed of $\alpha_1\beta_2\gamma_1$ subunits (second column) and $\alpha_1\beta_2$ subunits (third column)

Each value represents the mean $\pm$ S.E. of at least four oocytes from at least two oocyte batches.

Compound	I_{GABA} (EC$_{5-10}$) Potentiation	
	$\alpha_1\beta_2\gamma_1$	$\alpha_1\beta_2$
	%	
Triazolam	82 $\pm$ 10*	−21 $\pm$ 4*
Clotiazepam	53 $\pm$ 8*	−14 $\pm$ 2*
Midazolam	43 $\pm$ 4*	−20 $\pm$ 5*
CGS 20625	42 $\pm$ 4*	20 $\pm$ 5*
CGS 9896	24 $\pm$ 3*	16 $\pm$ 7
Diazepam	20 $\pm$ 3*	2 $\pm$ 2
Zolpidem	13 $\pm$ 3*	−5 $\pm$ 3
Bretazenil	10 $\pm$ 1*	−1 $\pm$ 5
DMCM	8 $\pm$ 4	4 $\pm$ 2
Cl 218,872	6 $\pm$ 5	−10 $\pm$ 1*
Clobazam	5 $\pm$ 2	7 $\pm$ 3
Flumazenil	4 $\pm$ 3	13 $\pm$ 5
Ru 33203	2 $\pm$ 2	−5 $\pm$ 5
PK 9084	−3 $\pm$ 3	−6 $\pm$ 4
Flurazepam	−5 $\pm$ 3	26 $\pm$ 4*
L-655,708	−7 $\pm$ 2	−8 $\pm$ 5
Ru 31719	−7 $\pm$ 3	−10 $\pm$ 5
Ru 33356	−7 $\pm$ 3	−2 $\pm$ 3
Ru 32698	−8 $\pm$ 2	−5 $\pm$ 2
Flunitrazepam	−11 $\pm$ 2*	−9 $\pm$ 5
PK 8165	−16 $\pm$ 2*	−14 $\pm$ 2*

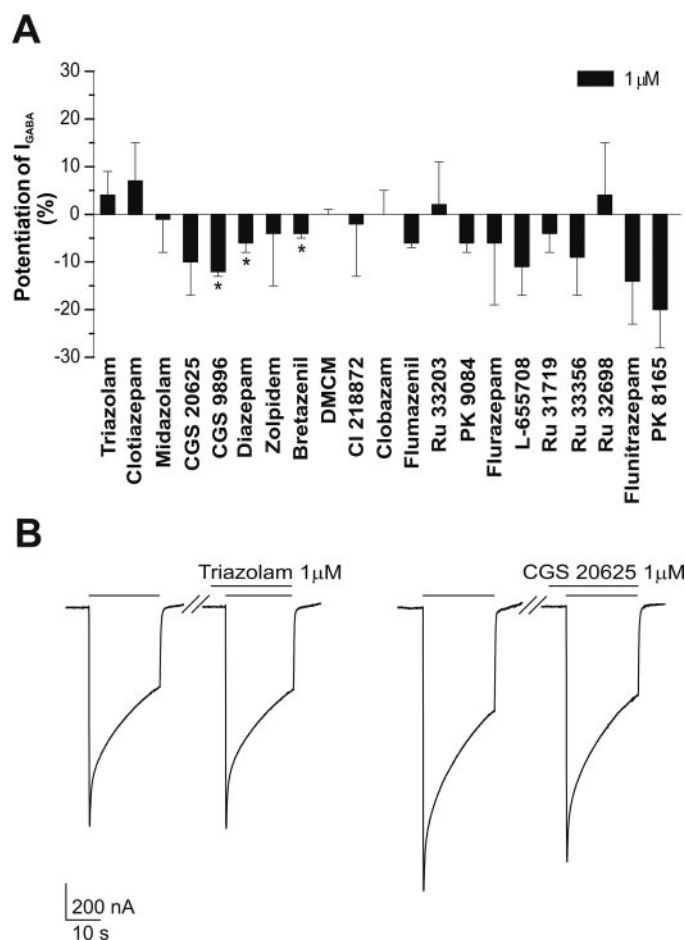* Difference from zero was calculated by ANOVA.

Fig. 3. A, modulation of chloride currents through GABA$_A$ receptors composed of $\alpha_1\beta_2\gamma_1$ subunits at 1 mM GABA by 1 μM concentration of the indicated compounds. ∗, statistically significant differences from 0 ($p < 0.05$, t test by ANOVA). B, typical enhancement of I_{GABA} through $\alpha_1\beta_2\gamma_1$ channels induced by 1 mM GABA in the absence (control, left traces) and presence of 1 μM triazolam and 1 μM CGS 20625.

et al., 2002). To clarify whether the effects observed were caused by $\alpha_1\beta_2\gamma_1$ or by $\alpha_1\beta_2$-comprising receptors, we analyzed the effect of these compounds on GABA$_A$ channels composed of $\alpha_1\beta_2$ subunits. With the exception of CGS 20625 ($+20 \pm 5\%$), CGS 9896 ($+16 \pm 7\%$), flumazenil ($+13 \pm 5\%$), and flurazepam ($+26 \pm 4\%$), all compounds (1 μM) were either inefficient in enhancing I_{GABA} or even induced significant inhibition (triazolam, clotiazepam, midazolam, Cl 218,872, and PK 8165; Table 1).

For the most potent stimulators of the $\alpha_1\beta_2\gamma_1$ receptors triazolam, clotiazepam, and midazolam, we also analyzed the inhibition of I_{GABA} in oocytes expressing only $\alpha_1\beta_2$ subunits at higher (10 μM) concentrations. Triazolam inhibited the GABA-induced chloride flux in $\alpha_1\beta_2$ receptors by $-33 \pm 4\%$ ($n = 12$), clotiazepam by $-30 \pm 8\%$ ($n = 7$), and midazolam by $-31 \pm 9\%$ ($n = 5$) (experiments not shown).

Comparing the Effects of Benzodiazepine Site Ligands on $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2\gamma_{2s}$ Receptors. Triazolam, clotiazepam, midazolam, and CGS 20625 were subsequently analyzed in more detail by comparing their effects on $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2\gamma_{2S}$ receptors. Figure 4 illustrates the concentration-dependence of the enhancement of the currents (EC$_{5-10}$) by triazolam, clotiazepam, and midazolam. The EC$_{50}$ value was determined by fitting the concentration-effect data to the Hill equation. Triazolam enhanced the maximum chloride current of $\alpha_1\beta_2\gamma_1$ receptors by 85% while displaying the highest potency (EC$_{50} \approx 90$ nM) of all tested benzodiazepines. Clotiazepam elicited an enhancement of the GABA response of approximately 170% but had a 19-fold lower potency (EC$_{50} \approx$ 1.7 μM) than triazolam. Midazolam was more potent than clotiazepam (EC$_{50} \approx 1.2$ μM) but was 13 times less potent than triazolam, with a maximum enhancement (92%) comparable with triazolam.

A comparison with the concentration-response data obtained on GABA$_A$ channels containing γ_{2S} subunits reveals a 3-fold higher efficiency of triazolam, a 1.5-fold higher efficiency of clotiazepam, and a 3.7-fold higher efficiency of midazolam on $\alpha_1\beta_2\gamma_{2S}$ receptors. The ratio of the EC$_{50}$ values for triazolam, clotiazepam, and midazolam (EC$_{50/\gamma1}$/EC$_{50/\gamma2S}$) reflect 4-, 9-, and 8-fold lower potencies of these compounds for $\alpha_1\beta_2\gamma_1$ receptors, respectively (Table 2). The apparent EC$_{50}$ values, maximum enhancement, and the corresponding ratios for the benzodiazepines tested are given in Table 2.

Modulation of I_{GABA} by Triazolam, Clotiazepam, and Midazolam at Different GABA Concentrations. To gain insight into the mechanism of I_{GABA} enhancement, we studied the GABA dose-effect curves in the absence and presence of the modulators. The results are shown in Fig. 5, A to C. In control, the mean EC$_{50}$ value for GABA was 39 ± 3 μM in $\alpha_1\beta_2\gamma_1$ receptors and 50 ± 3 μM in $\alpha_1\beta_2\gamma_{2S}$ receptors. The three benzodiazepine receptor ligands shifted the dose-effect curves to the left without affecting the maximal response (Fig. 3A). It is noteworthy that the drug-induced shift was more pronounced for $\alpha_1\beta_2\gamma_{2S}$ than for $\alpha_1\beta_2\gamma_1$ receptors, reflecting the higher efficiency of these ligands on $\alpha_1\beta_2\gamma_{2S}$.

Effect of the Pyrazolopyridine CGS 20625 on $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2\gamma_{2S}$ Receptors. CGS 20625 elicited maximum enhancement of chloride currents through $\alpha_1\beta_2\gamma_1$ receptors of approximately 645%, with a half-maximal enhancement occurring at approximately 20 μM (Fig. 6B). CGS 20625 thus represents a low potency but highly efficient positive modulator of $\alpha_1\beta_2\gamma_1$ receptors. This compound enhanced the GABA response of $\alpha_1\beta_2\gamma_{2S}$ receptors with comparable efficiency ($I_{max/\gamma2}$/$I_{max/\gamma1} \approx 1.12$) and was less efficient on $\alpha_1\beta_2$ receptors (Fig. 6B). At higher CGS 20625 concentrations (≥ 300 μM), we observed weaker enhancement of the GABA-induced chloride flux than at 100 μM for all subunit compositions (Fig. 6B).
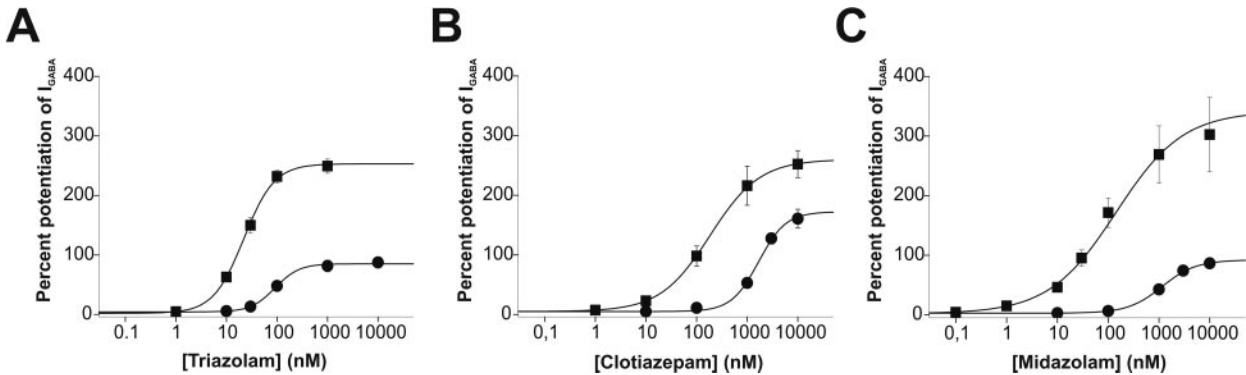
Fig. 4. Concentration-effect curves for triazolam, clotiazepam, and midazolam on $\alpha_1\beta_2\gamma_1$ (●) and $\alpha_1\beta_2\gamma_{2S}$ receptors (■) using an EC$_{5-10}$ GABA concentration (EC$_{50}$ values are given in Table 2). Data points represent means $\pm$ S.E. from at least four oocytes from ≥ 2 batches.

TABLE 2
Potency and efficiency of triazolam, midazolam, clotiazepam, and CGS 20625 for GABA$_A$ receptors composed of $\alpha_1\beta_2\gamma_1$ or $\alpha_1\beta_2\gamma_{2S}$ subunits
The maximum enhancement (percentage of control) and EC$_{50}$ values were calculated by fitting the data points to a standard concentration-response curve. Rightmost column represents ratios between EC$_{50}$ values of $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2\gamma_{2S}$ receptors.

Compound	$\alpha_1\beta_2\gamma_1$		$\alpha_1\beta_2\gamma_{2s}$		EC$_{50}(\gamma_1)$/EC$_{50}(\gamma_{2S})$
	EC$_{50}$	Maximum potentiation	EC$_{50}$	Maximum potentiation	
	nM	*%*	*nM*	*%*	
Triazolam	92 ± 17	85 ± 7	22 ± 3	253 ± 12	4.18
Midazolam	1150 ± 259	92 ± 8	143 ± 88	342 ± 64	8.04
Clotiazepam	1681 ± 432	172 ± 24	184 ± 88	260 ± 27	9.14
CGS 20625	$23{,}712 \pm 6835$	645 ± 55	$11{,}220 \pm 722$	724 ± 25	2.11

Effect of Flumazenil on $\alpha_1\beta_2\gamma_1$ Receptors. Flumazenil is a ligand of the BZ binding site of $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptors and competitively inhibits the enhancement of GABA-induced chloride currents by benzodiazepine agonists (Wafford et al., 1993). It was therefore interesting whether this compound would also inhibit the effects of triazolam and clotiazepam on $\alpha_1\beta_2\gamma_1$ receptors.

Figure 7, A and B (left), illustrates the inhibition by fluma-

zenil of triazolam- or clotiazepam-induced I_{GABA} enhancement in $\alpha_1\beta_2\gamma_{2S}$ receptors. As shown on the right, the effects of triazolam or clotiazepam on $\alpha_1\beta_2\gamma_1$ receptors were not inhibited by 1 μM flumazenil. Moreover, we were unable to study possible antagonistic effects at higher concentrations, because flumazenil induced significant enhancement of I_{GABA} in oocytes expressing $\alpha_1\beta_2\gamma_1$ subunits at 10 (22 $\pm$ 2%, $n = 4$) and 100 μM (64 $\pm$ 8%, $n = 4$; Fig. 7C).
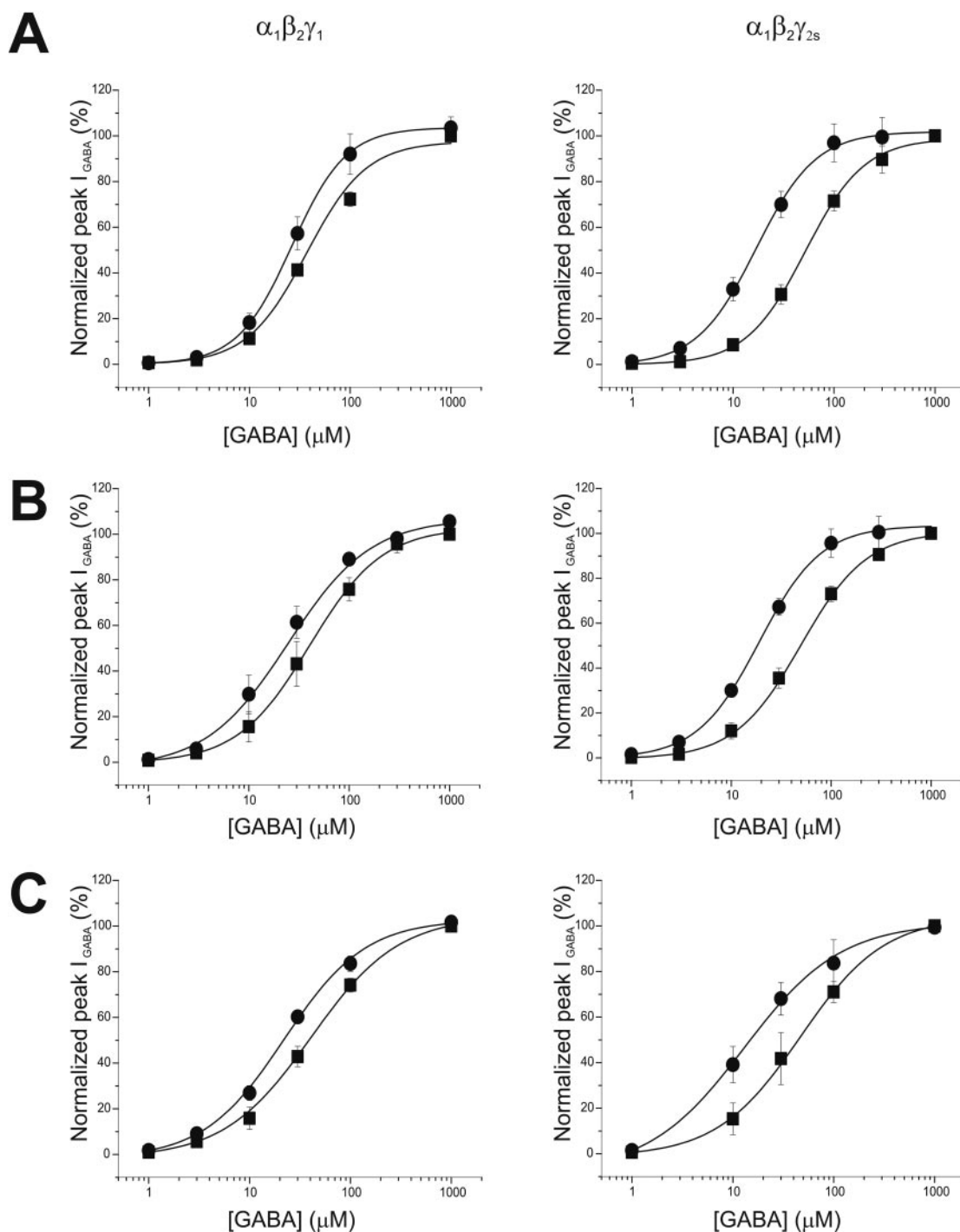

Fig. 5. Modulation of the GABA concentration-response curve of $\alpha_1\beta_2\gamma_1$ (left) and $\alpha_1\beta_2\gamma_{2S}$ receptors (right) by 1 μM triazolam (A), 10 μM clotiazepam (B), and 10 μM midazolam (C). The corresponding mean EC$_{50}$ values were 37 $\pm$ 2 μM (control), 26 $\pm$ 4 μM (triazolam) for $\alpha_1\beta_2\gamma_1$ and 52 $\pm$ 7 μM (control), and 17 $\pm$ 3 μM (triazolam) for $\alpha_1\beta_2\gamma_{2S}$ (A); 40 $\pm$ 8 μM (control), 25 $\pm$ 4 μM (clotiazepam) for $\alpha_1\beta_2\gamma_1$ and 50 $\pm$ 5 μM (control), and 19 $\pm$ 3 μM (clotiazepam) for $\alpha_1\beta_2\gamma_{2S}$ (B); and 41 $\pm$ 6 μM (control), 22 $\pm$ 2 μM (midazolam) for $\alpha_1\beta_2\gamma_1$ and 47 $\pm$ 11 μM (control), and 13 $\pm$ 6 μM (midazolam) for $\alpha_1\beta_2\gamma_{2S}$ (C).

Discussion

In the present study, we made use of a fast and automated perfusion technique (Fig. 1) to test a selection of 21 modulators on GABA$_A$ receptors composed of $\alpha_1\beta_2\gamma_1$ subunits. Solution exchange occurred between 100 and 250 ms (see *Materials and Methods* and Fig. 1C), which reduced the effects of desensitization on peak current detection compared with conventional bath perfusion.

I_{GABA} of $\alpha_1\beta_2\gamma_1$ Subunit Receptors Is Enhanced by Some Benzodiazepines and the Pyrazolopyridine CGS 20625. The 21 compounds tested comprised benzodiazepines and representatives of other structural classes of ligands of the BZ binding site of GABA$_A$ receptors. To determine the most potent modulators of $\alpha_1\beta_2\gamma_1$ subunit receptors, we first tested the compounds at a concentration of 1 μM. Of the 21 compounds, six induced an enhancement of the GABA response (EC$_{5-10}$) by more than 20% with the following order of potency: triazolam > clotiazepam > midazolam > CGS 20625 > CGS 9896 > diazepam. Zolpidem and bretazenil induced a small enhancement ($\approx$13 and 10%, respectively). The effects of DMCM, Cl 218,872, clobazam, flumazenil, Ru 33203, PK 9084, flurazepam, L-655,708, Ru 31719, Ru 33356, and Ru 32698 were not significantly different from control. In contrast, flunitrazepam and PK 8165 significantly inhibited

the GABA-induced chloride flux. Application of higher concentrations (10 and 100 μM) revealed a moderate enhancement at high concentrations (low-potency modulation) by bretazenil, DMCM, flumazenil, Ru 33203, Ru 32698, and flunitrazepam. All compounds were tested in the absence of GABA (data not shown). None of them induced measurable currents, even at high concentrations (up to 100 μM). Thus, the 1,4-benzodiazepines triazolam and midazolam, the 1,4-thienodiazepine clotiazepam, and the pyrazolopyridine CGS 20625 seemed to be the most promising candidates for further detailed analysis.

Contribution of the γ_1 Subunit to the Enhancement of I_{GABA}. On injection of *X. laevis* oocytes with α, β, and γ subunits, not only are receptors containing all three subunits formed but possibly also receptors composed of α and β subunits only (Boileau et al., 2002). To investigate whether the observed drug effects were caused by effects on $\alpha_1\beta_2\gamma_1$ receptors or could also be explained by effects on $\alpha_1\beta_2$ receptors, the effects of drugs on the latter receptors were also investigated. A comparison of drug effects on $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2$ receptors revealed that the γ_1 subunit was essential for enhancement of I_{GABA} by triazolam, clotiazepam, and midazolam (Table 1 and Fig. 2), because these compounds at 1 μM concentration induced a significant inhibition by $-21 \pm$
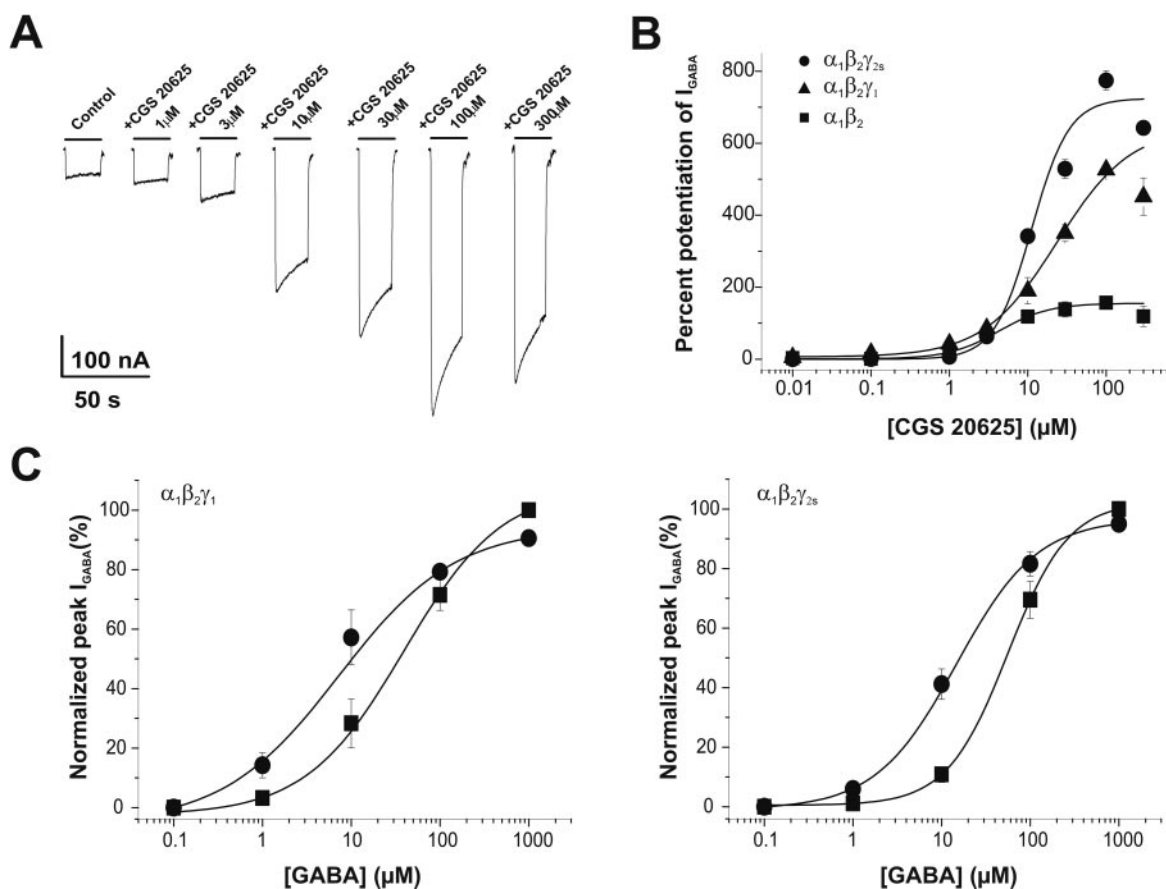

Fig. 6. Modulation of I_{GABA} by CGS 20625. A, typical I_{GABA} recordings illustrating concentration-dependent modulation of GABA-elicited chloride currents through $\alpha_1\beta_2\gamma_1$-containing receptors. B, concentration-effect curves for CGS 20625 on $\alpha_1\beta_2\gamma_{2S}$ ($\bullet$), $\alpha_1\beta_2\gamma_1$ ($\blacktriangle$), and $\alpha_1\beta_2$ ($\blacksquare$) receptors. EC$_{50}$ values and corresponding Hill coefficient were the following: 11.2 $\pm$ 0.7 μM, n_H = 1.8 $\pm$ 0.1 ($\bullet$); 23.7 $\pm$ 6.8 μM, n_H = 0.9 $\pm$ 0.1 ($\blacktriangle$); and 4.3 $\pm$ 1.2 μM and n_H = 1.4 $\pm$ 0.2 ($\blacksquare$), respectively. Each data point represents mean $\pm$ S.E. from at least four oocytes and $\geq$2 batches. I_{GABA} at 300 μM (open symbols) were excluded from the fit. C, modulation of the GABA concentration response of $\alpha_1\beta_2\gamma_1$ (left) and $\alpha_1\beta_2\gamma_2$ receptors (right) by 100 μM CGS 20625. The corresponding EC$_{50}$ values were 39 $\pm$ 16 μM (control) and 7 $\pm$ 2 μM (CGS 20625) in $\alpha_1\beta_2\gamma_1$ receptors and 56 $\pm$ 14 μM (control) and 15 $\pm$ 3 μM (CGS 20625) in $\alpha_1\beta_2\gamma_{2S}$ receptors.

4% ($n = 12$, $p < 0.05$), ($-14 \pm 2\%$, $n = 6$), and ($-20 \pm 5\%$, $n = 6$), respectively, of I_{GABA} through $\alpha_1\beta_2$ subunit receptors. In addition, the effects of several other drugs were clearly different in $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2$ receptors. Thus, CGS 20625, CGS 9896, diazepam, zolpidem, and bretazenil exhibited an enhancement of I_{GABA} that was higher in $\alpha_1\beta_2\gamma_1$ than in $\alpha_1\beta_2$ receptors, supporting the importance of the γ_1 subunit for the effects observed. Finally, Cl 218,872 and flurazepam exhibited effects in opposite direction in $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2$ receptors, again supporting the conclusion that the drug effects observed on injection of oocytes with α_1, β_2, and γ_1 subunits were predominantly caused by $\alpha_1\beta_2\gamma_1$ receptors (Table 1).

Our finding that triazolam, clotiazepam, and midazolam significantly inhibit I_{GABA} through $\alpha_1\beta_2$ receptors suggests that we might have underestimated the enhancement of I_{GABA} in $\alpha_1\beta_2\gamma_1$ receptors by these drugs because of simultaneously occurring inhibition of $\alpha_1\beta_2$ subunit receptors. The differential effects of various BZ binding site ligands on receptors composed of $\alpha_1\beta_2$ subunits are highly interesting by themselves and significantly extend previous evidence for the existence of a low-affinity benzodiazepine binding site at $\alpha\beta$ receptors (Wafford et al., 1993; Thomet et al., 1999; Walters et al., 2000).

To determine the potency and efficiency (maximum ability to enhance the GABA EC$_{5-10}$ response of the three most potent BZ; Table 2), we studied their concentration effect for receptors composed of $\alpha_1\beta_2\gamma_1$ subunits (Fig. 3). The 1,4-benzodiazepine triazolam displayed the highest potency (EC$_{50} \approx$ 90 nM), followed by midazolam (EC$_{50} \approx 1.2$ μM) and the 1,4-thienodiazepine clotiazepam (EC$_{50} \approx 1.7$ μM, Table 2). A comparison of the concentration-effect curves for these benzodiazepine-type ligands with receptors composed of $\alpha_1\beta_2\gamma_{2S}$ receptors revealed a significantly lower efficiency and potency for γ_1-containing receptors (Fig. 3 and Table 2).
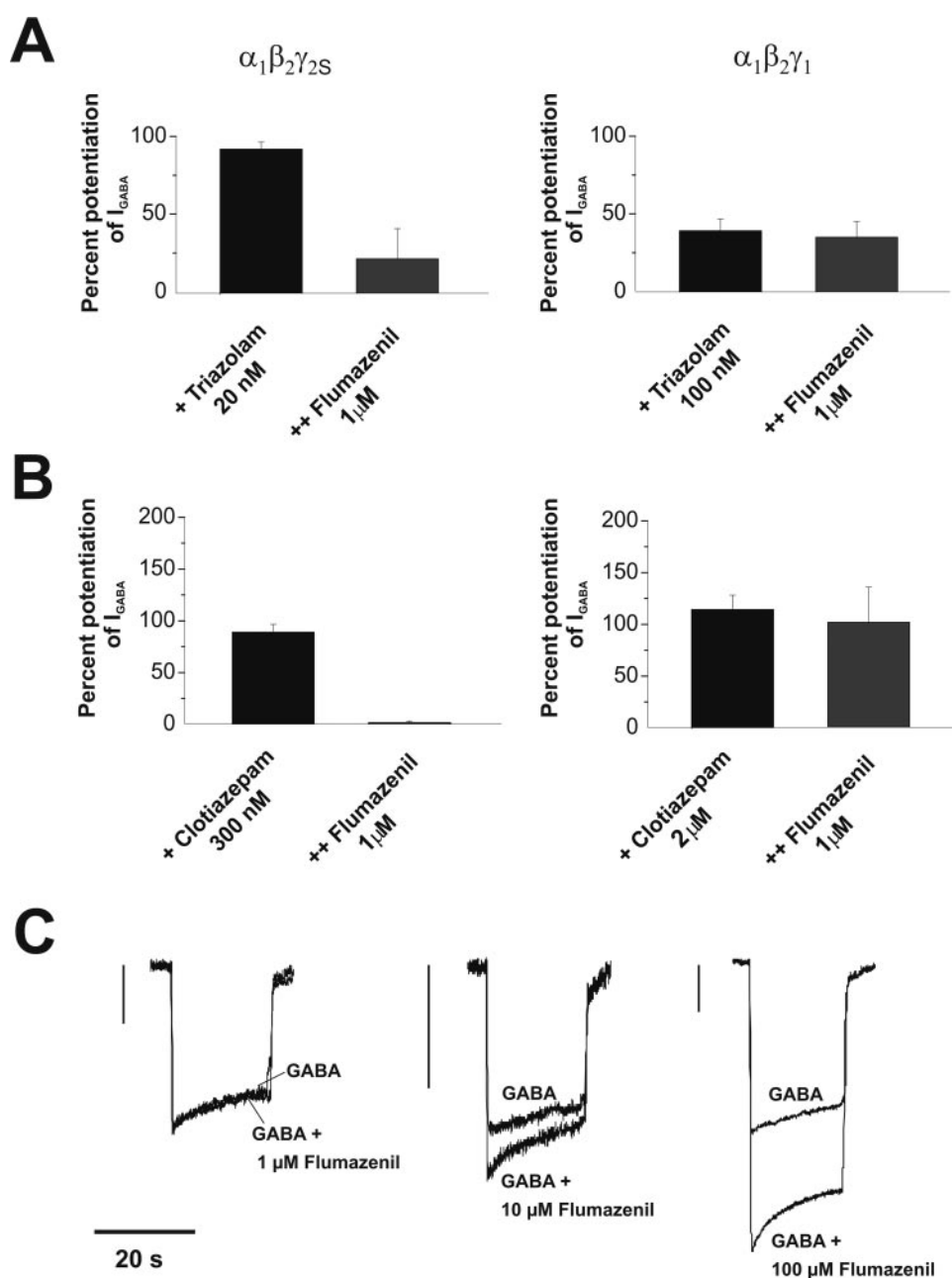

Fig. 7. A and B, effect of flumazenil on the enhancement of I_{GABA} by triazolam and clotiazepam. The left graphs illustrates the enhancement of I_{GABA} through $\alpha_1\beta_2\gamma_{2s}$ receptors by triazolam and clotiazepam in the absence and presence of flumazenil. The right graphs illustrate the lack of inhibition of I_{GABA} through $\alpha_1\beta_2\gamma_1$ receptors. C, typical I_{GABA} through $\alpha_1\beta_2\gamma_1$ receptors in the absence (GABA) and presence of the indicated concentrations of flumazenil.

The Pyrazolopyridine CGS 20625 Displays the Highest Efficiency in Enhancing GABA-Induced Chloride Flux of $\alpha_1\beta_2\gamma_1$ Subunit Receptors. CGS 20625 was identified as the most efficient compound in terms of maximum enhancement of the GABA-induced chloride currents through $\alpha_1\beta_2\gamma_1$ subunit receptors (Fig. 6). This compound induced a maximum enhancement of $645 \pm 55\%$ greater than control, which is approximately 3.75-fold the enhancement achieved with clotiazepam ($172 \pm 24\%$ greater than control), and more than 7 times the enhancement induced by midazolam ($92 \pm 8\%$ greater than control) or by triazolam ($85 \pm 7\%$ above control), respectively (Fig. 3). CGS 20625, however, had a potency for $\alpha_1\beta_2\gamma_1$ receptors (EC$_{50} \approx 20$ μM) approximately 200 times lower than that of triazolam and 10 to 20 times lower than that of the other benzodiazepines (Table 2).

A closer inspection of the subunit composition specificity of CGS 20625 action revealed that this drug potentiates $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2\gamma_{2S}$ subunit receptors to an almost similar extent (Fig. 6B). However, a significantly lower efficiency on $\alpha_1\beta_2$ receptors revealed an essential role of a γ subunit.

CGS 20625 at 300 μM caused less enhancement than at 100 μM in $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_1$, and $\alpha_1\beta_2\gamma_{2S}$ subunit receptors (Fig. 6B), suggesting that this compound might inhibit chloride currents at high concentrations. Similar behavior was shown previously for the action of another pyrazolopyridine (tracazolate) on GABA$_A$ channels (Thompson et al., 2002).

CGS 20625 thus represents a low-potency (EC$_{50} \approx 20$ μM) but high-efficiency modulator of $\alpha_1\beta_2\gamma_1$ and $\alpha_1\beta_2\gamma_{2S}$ subunit-containing receptors (Fig. 6B). This compound was almost not selective for either γ_1 or γ_2 subunits. $\alpha_1\beta_2$ Subunit receptors were, however, stimulated to a significantly lesser extent ($\approx$160%) compared with $\alpha_1\beta_2\gamma_{1/2S}$ subunit receptors (640–730%) (Fig. 6B).

The competitive antagonist flumazenil (1 μM) inhibited I_{GABA} enhancement of $\alpha_1\beta_2\gamma_{2S}$ receptors but failed to affect the enhancement of I_{GABA} through $\alpha_1\beta_2\gamma_1$ receptors by triazolam and clotiazepam (Fig. 7, right). These data suggest that flumazenil exhibits either no or a very low affinity for the BZ binding site of $\alpha_1\beta_2\gamma_1$ receptors, or that flumazenil interacts with a binding site different from that for triazolam and clotiazepam at these receptors. The first conclusion is consistent with the observation that the affinity of flumazenil for its binding site was reduced approximately 1000-fold in GABA$_A$ receptors in which phenylalanine 77 of the γ_2 subunit was mutated to the corresponding residue (isoleucine) of the γ_1 subunit (γ_2F77I; Buhr et al., 1997; Wingrove et al., 2002; Ogris et al., 2004). At higher concentrations, flumazenil displayed properties of a low-affinity agonist on $\alpha_1\beta_2\gamma_1$-receptors (10 μM potentiated I_{GABA} by $22 \pm 2\%$ and 100 μM by $64 \pm 9\%$).

In addition to flumazenil, the affinities of bretazenil, L-655,708, DMCM, zolpidem, Cl 218,872, and PK 8165 were drastically reduced in receptors containing the γ_2F77I point mutation (Ogris et al., 2004), as measured by [^{3}H]flunitrazepam binding studies. A low affinity of these compounds for $\alpha_1\beta_2\gamma_1$ receptors could have contributed to their small effects on these receptors observed in the present study. In contrast, replacement of phenylalanine (γ_2F77) by the corresponding isoleucine of the γ_1 subunit only weakly (2- to 7-fold) reduced the affinity of the classic 1,4-benzodiazepines, the 1,4-thienodiazepine clotiazepam, the 1,5-benzodiazepine clobazam, or the pyrazoloquinoline CGS 9896 (Ogris et al., 2004). The

small (4- to 9-fold) reduction in potency of triazolam, midazolam, and clotiazepam for enhancing $\alpha_1\beta_2\gamma_1$ compared with $\alpha_1\beta_2\gamma_2$ receptors could be explained by a reduced apparent affinity of these compounds for $\alpha_1\beta_2\gamma_1$ receptors underlining the importance of Phe77 for high-affinity BZ binding (Buhr et al., 1997). For other compounds such as flunitrazepam (1 μM), we observed significant inhibition of I_{GABA} in $\alpha_1\beta_2\gamma_1$ receptors, indicating the importance of additional amino acids for drug binding and gating.

The different efficiency of triazolam, midazolam, clotiazepam, and CGS 20625 are explained by the different amounts of shifts of the GABA concentration-effect curves (Fig. 5, A–C). Larger shifts induced on $\alpha_1\beta_2\gamma_{2S}$ receptors reflect the higher apparent efficiency.

In summary we systematically investigated 21 ligands of the BZ binding site from chemically distinct classes to obtain insight in the pharmacological profile of GABA$_A$ receptors comprising a γ_1 subunit. Triazolam was identified as a high-potency and CGS 20625 as a high-efficiency modulator of this receptor subtype. Different potencies of triazolam, midazolam, clotiazepam, and CGS 20625 can be explained by different shifts of the GABA dose-effect curve, reflecting different apparent affinities of these compounds.

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