Pharmacological Properties of GABA_\textsubscript{A} Receptors Containing \gamma_1 Subunits

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ABSTRACT

GABA_\textsubscript{A} receptors composed of \alpha_1, \beta_2, \gamma_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_2, \beta_2, \gamma_1-GABA_\textsubscript{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, 4-(4-ethyl-piperidinyl)-quinoline (PK 9084), flurazepam, ethyl-7-methoxy-11,12,13a-tetrahydro-9-oxo-9H-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 33565), and 6-ethyl-7-methoxy-5-methylimidazo[1,2-a]pyrimidin-2-yl)phenylmethanone (Ru 32698) (1 \mu M 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_2, \beta_2, \gamma_1 and \alpha_2, \beta_2, \gamma_2S receptors. The potency and efficiency of these compounds for modulating I_{GABA} was smaller for \alpha_2, \beta_2, \gamma_2S than for \alpha_2, \beta_2, \gamma_1 receptors, and their effects on \alpha_2, \beta_2, \gamma_1 could not be blocked by flumazenil. CGS 20625 displayed the highest efficiency by enhancing at 100 \mu M I_{GABA} (\alpha_2, \beta_2, \gamma_1) by 775 \pm 17\% versus 526 \pm 14\% I_{GABA} (\alpha_2, \beta_2, \gamma_2S) and 157 \pm 17\% I_{GABA} (\alpha_2, \beta_2, \gamma_2S) (p < 0.05). These data provide new insight into the pharmacological properties of GABA_\textsubscript{A} receptors containing \gamma_1 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 interacting with the GABA_\textsubscript{A} receptor. GABA_\textsubscript{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_\textsubscript{A} receptor subunits have been cloned: \alpha_1–6, \beta_1–3, \gamma_1–3, \delta, \epsilon, \eta, \theta, \pi, \rho, \sigma, and \theta (Barnard et al., 1998; Simon et al., 2004). The major receptor subtype of the GABA_\textsubscript{A} receptor in adults consists of \alpha_1, \beta_2, and \gamma_2 subunits, and the most likely stoichiometry is two \alpha subunits, two \beta subunits, and one \gamma subunit (Sieghart and Sperk, 2002).

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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 (CGS 9896), dizepam, zolpidem, and bretazenil at 1 \mu M concentrations were able to significantly (>20\%) enhance I_{GABA} in \alpha_2, \beta_2, \gamma_1 receptors. Methyl-6,7-di-methoxy-4-ethyl-\beta-carboline-3-carboxylate, 3-methyl-[3-trifluoromethyl-phenyl]-1,2,4-triazolo[4,3-b]pyridazine (C1 218872), 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-11,12,13a-tetrahydro-9-oxo-9H-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 33565), and 6-ethyl-7-methoxy-5-methylimidazo[1,2-a]pyrimidin-2-yl)phenylmethanone (Ru 32698) (1 \mu M 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_2, \beta_2, \gamma_1 and \alpha_2, \beta_2, \gamma_2S receptors. The potency and efficiency of these compounds for modulating I_{GABA} was smaller for \alpha_2, \beta_2, \gamma_2S than for \alpha_2, \beta_2, \gamma_1 receptors, and their effects on \alpha_2, \beta_2, \gamma_1 could not be blocked by flumazenil. CGS 20625 displayed the highest efficiency by enhancing at 100 \mu M I_{GABA} (\alpha_2, \beta_2, \gamma_1) by 775 \pm 17\% versus 526 \pm 14\% I_{GABA} (\alpha_2, \beta_2, \gamma_2S) and 157 \pm 17\% I_{GABA} (\alpha_2, \beta_2, \gamma_2S) (p < 0.05). These data provide new insight into the pharmacological properties of GABA_\textsubscript{A} receptors containing \gamma_1 subunits and may aid in the design of specific ligands for this receptor subtype.

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The subunit composition determines the GABA sensitivity and the pharmacological properties of the GABA<sub>A</sub> 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<sub>2</sub> subunits modulate the BZ sensitivity of the receptor, suggesting that the BZ binding pocket is located at the interface between α and γ<sub>2</sub> (Sigel, 2002; Ernst et al., 2003). There is clear evidence that substitution of the γ<sub>2</sub> subunit by either γ<sub>1</sub> or γ<sub>2</sub> significantly alters the sensitivity for BZ (Hevers and Luddens, 1998).

In contrast to the γ<sub>2</sub> subunit, which is ubiquitously expressed in the central nervous system, the γ<sub>1</sub> 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 (centralateral and medial nuclei) (Pirker et al., 2000; Korpi et al., 2002). Compounds selectively interacting with receptors containing γ<sub>1</sub> subunits thus might have a substantial clinical potential.

Compared with receptors containing γ<sub>2</sub> subunits, little is known about the pharmacological profile of GABA<sub>A</sub> channels composed of α<sub>1</sub>, β<sub>2</sub>, and γ<sub>1</sub> subunits. Ymer et al. (1990) observed a loss of sensitivity for the benzodiazepine agonist Ro 15-1788 and the inverse agonist methyl-6,7-dimethoxy-4-ethyl-b-carboline-3-carboxylate (DMCC) when the γ<sub>1</sub> was substituted for γ<sub>2</sub> in α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> receptors. Negative modulatory effects of Ro 15-4513, β-CCM, and DMCC for GABA<sub>A</sub> receptors composed of α<sub>1</sub>γ<sub>1</sub>β<sub>2</sub> subunits are changed to positive modulatory effects in α<sub>1</sub>γ<sub>1</sub>β<sub>2</sub> 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<sub>1</sub>-containing receptors. Wafford et al. (1993) demonstrated a reduced enhancement of chloride currents through α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> by diazepam, clonazepam, and brezatilin compared with α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub> and a negative modulatory effect of zolpidem for α<sub>1</sub>β<sub>2</sub>γ<sub>1</sub> 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<sub>A</sub> receptors containing γ<sub>1</sub> 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<sub>1</sub>. Thus, the relative efficacies of these compounds for αβγ<sub>1</sub> are not comparable (Hevers and Luddens, 1998).

In the present study, we analyzed the modulation of α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> receptors expressed in Xenopus <i>laevis</i> 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<sub>1</sub>-containing receptors.

### Materials and Methods

**Chemicals.** Compounds were obtained from the following sources: flunitrazepam (7-nitro-1,3-dihydro-1-methyl-5-o-fluoro phenyl-2H-1,4-benzodiazepin-2-one), diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one), flurazepam (7-chloro-1,3-dihydro-1-ethylaminodiethyl-5-o-fluorophenyl-2H-1,4-benzodiazepin-2-one), midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5-a][1,4]benzodiazepine), Ro 15-1788, and brezatilin [6-butyryl]-8-bromoo-11,12,13,13a-tetrahydro-9-oxo-9H-imidazo[1,5-a][1,4]benzodiazepine-1-carboxylate) were from Hoffmann La Roche (Basel, Switzerland); t-657,708 was purchased from Tocris Cookson Inc. (Bristol, UK); clotiazepam [5-(2-chlorophenyl)-7-ethyl-1,3-dihydro-1-methyl-2H-thieno[2,3-e][1,4]diazepin-2-one] was from Troponwerk (Koln, Germany); clobazam [7-chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4(3H,5H)-dione) was from sanofi-aventis (Bridgewater, NJ); triazolam [8-chloro-6-(2-chlorophenyl)-1-methyl-4H-1,2,4-triazolo[4,3-a][1,4]benzodiazepine] was from Sigma (Vienna, Austria); DMCM was from Ferrosan (Soeborg, Denmark); CGS 9986 and CGS 20625 were from Novartis (Basel, Switzerland); zolpidem [N,N6,6-trimethyl-2-(4-methylphenyl)imidazol[1,2-c]pyridine-3-acetamide] was from Synthalabo Recherche (Bagnex, France); CI 218,872 was from American Cyanamide Comp. (Wayne, NJ); Ru 31719, Ru 32698, Ru 33203, and Ru 33256 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<sub>A</sub> Receptors.** <i>X. laevis</i> oocytes were prepared and injected as described previously (Grabner et al., 1996). Female <i>X. laevis</i> (Nasco, Fort Atkinson, WI) were anesthetized by exposing them for 15 min to a 0.2% MS-222 (methylene 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 IA, 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, Steinfink, Germany). To ensure expression of the γ subunit in the case of α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> and α<sub>1</sub>β<sub>2</sub>γ<sub>3</sub> receptors, cRNAs were mixed in a ratio of 1:1:10, and for receptors containing only α<sub>1</sub> and β<sub>2</sub> 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<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 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 <i>X. laevis</i> oocytes were performed in a small (<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 Digida Data 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<sub>1</sub> channels in <i>X. laevis</i> 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 current traces of K<sub>1</sub> currents of 80 mM a-tocopherol (−80 to +20 mV) for a current decay of 10 mV potassium at a speed of 1 m/s is shown on the right in Fig. 1B. A...
mean current decline time $t_{10-90\%}$ of $140.8 \pm 17.5 \text{ ms (n = 7)}$ was estimated.

To elicit GABA-induced chloride currents ($I_{\text{GABA}}$), the chamber was perfused with $120 \mu l$ of GABA-containing solution at the same volume rate (1 ml/s). The rise time of $I_{\text{GABA}}$ 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 ml/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).

Analyzing Concentration-Response Curves. Enhancement of chloride currents by modulators of the GABA$_\alpha$ 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 $\mu M$) was determined at the beginning of each experiment.

Enhancement of the chloride current ($I_{\text{GABA}}$) was defined as $(I_{\text{GABA} + \text{Comp}}/I_{\text{GABA}}) - 1$, where $I_{\text{GABA} + \text{Comp}}$ is the current response in the presence of a given compound, and $I_{\text{GABA}}$ is the control GABA current. To measure the sensitivity of the GABA$_\alpha$ receptor for a...
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])$^n$), where EC$_{50}$ is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50%, and $n$ is the Hill coefficient. Data are given as mean ± 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), $\beta$-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 (A), 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, CI 218,872, clotiazepam, 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 “sympathetic 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 $I_{\text{GABA}}$. 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 $\gamma$ 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).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$I_{\text{GABA}}$/EC$_{5-10}$ Potentiation</th>
<th>$\alpha_1\beta_2\gamma_1$</th>
<th>$\alpha_1\beta_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triazolam</td>
<td>82 ± 10*</td>
<td>-21 ± 4*</td>
<td></td>
</tr>
<tr>
<td>Clotiazepam</td>
<td>53 ± 8*</td>
<td>-14 ± 2*</td>
<td></td>
</tr>
<tr>
<td>Midazolam</td>
<td>43 ± 4*</td>
<td>-20 ± 5*</td>
<td></td>
</tr>
<tr>
<td>CGS 20625</td>
<td>42 ± 4*</td>
<td>20 ± 5*</td>
<td></td>
</tr>
<tr>
<td>CGS 9896</td>
<td>24 ± 3*</td>
<td>16 ± 7</td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>20 ± 3*</td>
<td>2 ± 3</td>
<td></td>
</tr>
<tr>
<td>Zolpidem</td>
<td>13 ± 3*</td>
<td>-5 ± 3</td>
<td></td>
</tr>
<tr>
<td>Bretazenil</td>
<td>10 ± 1*</td>
<td>-1 ± 5</td>
<td></td>
</tr>
<tr>
<td>DMCM</td>
<td>8 ± 4</td>
<td>4 ± 2</td>
<td></td>
</tr>
<tr>
<td>CI 218,872</td>
<td>6 ± 5</td>
<td>-10 ± 1*</td>
<td></td>
</tr>
<tr>
<td>Clobazam</td>
<td>5 ± 2</td>
<td>7 ± 3</td>
<td></td>
</tr>
<tr>
<td>Flumazenil</td>
<td>4 ± 3</td>
<td>13 ± 5</td>
<td></td>
</tr>
<tr>
<td>Ru 33203</td>
<td>2 ± 2</td>
<td>-5 ± 4</td>
<td></td>
</tr>
<tr>
<td>Flurazepam</td>
<td>-7 ± 1*</td>
<td>-6 ± 4</td>
<td></td>
</tr>
<tr>
<td>L-655,708</td>
<td>-7 ± 2*</td>
<td>-8 ± 5</td>
<td></td>
</tr>
<tr>
<td>Ru 31719</td>
<td>-7 ± 3*</td>
<td>-10 ± 5</td>
<td></td>
</tr>
<tr>
<td>Ru 33356</td>
<td>-7 ± 3*</td>
<td>-2 ± 3</td>
<td></td>
</tr>
<tr>
<td>Ru 32698</td>
<td>-8 ± 3*</td>
<td>-5 ± 3</td>
<td></td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>-11 ± 2*</td>
<td>-9 ± 5</td>
<td></td>
</tr>
<tr>
<td>PK 8165</td>
<td>-16 ± 2*</td>
<td>-14 ± 2*</td>
<td></td>
</tr>
</tbody>
</table>

* Difference from zero was calculated by ANOVA.
et al., 2002). To clarify whether the effects observed were caused by α1β2γ1 or by α1β2-comprising receptors, we analyzed the effect of these compounds on GABA_A channels composed of α1β2 subunits. With the exception of CGS 20625 (+20 ± 5%), CGS 9896 (+16 ± 7%), flumazenil (+13 ± 5%), and flurazepam (+26 ± 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 α1β2γ1 receptors triazolam, clotiazepam, and midazolam, we also analyzed the inhibition of I_GABA in oocytes expressing only α1β2 subunits at higher (10 μM) concentrations. Triazolam inhibited the GABA-induced chloride flux in α1β2 receptors by −33 ± 4% (n = 12), clotiazepam by −30 ± 8% (n = 7), and midazolam by −31 ± 9% (n = 5) (experiments not shown).

Comparing the Effects of Benzodiazepine Site Ligands on α1β2γ1 and α1β2γ2S Receptors. Triazolam, clotiazepam, midazolam, and CGS 20625 were subsequently analyzed in more detail by comparing their effects on α1β2γ1 and α1β2γ2S receptors. Figure 4 illustrates the concentration-dependence of the enhancement of the currents (EC50–10) by triazolam, clotiazepam, and midazolam. The EC50 value was determined by fitting the concentration-effect data to the Hill equation. Triazolam enhanced the maximum chloride current of α1β2γ1 receptors by 85% while displaying the highest potency (EC50 ≈ 90 nM) of all tested benzodiazepines. Clotiazepam elicited an enhancement of the GABA response of approximately 170% but had a 19-fold lower potency (EC50 ≈ 1.7 μM) than triazolam. Midazolam was more potent than clotiazepam (EC50 ≈ 2.1 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 γ2S 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 α1β2γ2S receptors. The ratio of the EC50 values for triazolam, clotiazepam, and midazolam (EC50γ1/EC50γ2S) reflect 4-, 9-, and 8-fold lower potencies of these compounds for α1β2γ1 receptors, respectively (Table 2). The apparent EC50 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 EC50 value for GABA was 39 ± 3 μM in α1β2γ1 receptors and 50 ± 3 μM in α1β2γ2S 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 α1β2γ1 receptors than for α1β2γ2S receptors, reflecting the higher efficiency of these ligands on α1β2γ2S.

Effect of the Pyrazolopyridine CGS 20625 on α1β2γ1 and α1β2γ2S Receptors. CGS 20625 elicited maximum enhancement of chloride currents through α1β2γ1 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 α1β2γ1 receptors. This compound enhanced the GABA response of α1β2γ2S receptors with comparable efficiency (I_max/α1β2γ1 ≈ 1.12) and was less efficient on α1β2γ2S 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 α1β2γ1 (●) and α1β2γ2S receptors (□) using an EC50, GABA concentration (EC50, values are given in Table 2). Data points represent means ± S.E. from at least four oocytes from ≥2 batches.](image)

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 γ1 (%)</th>
<th>EC50 γ2S (%)</th>
<th>EC50 γ1/EC50 γ2S (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triazolam</td>
<td>92 ± 17</td>
<td>85 ± 7</td>
<td>1.12</td>
</tr>
<tr>
<td>Midazolam</td>
<td>1150 ± 259</td>
<td>92 ± 8</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>Clotiazepam</td>
<td>1681 ± 432</td>
<td>172 ± 24</td>
<td>143 ± 8</td>
</tr>
<tr>
<td>CGS 20625</td>
<td>23,712 ± 6835</td>
<td>645 ± 55</td>
<td>11,220 ± 722</td>
</tr>
</tbody>
</table>
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 flumazenil of triazolam- or clotiazepam-induced $I_{\text{GABA}}$ enhancement in $\alpha_1\beta_2\gamma_2$ 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 $\mu$M flumazenil. Moreover, we were unable to study possible antagonistic effects at higher concentrations, because flumazenil induced significant enhancement of $I_{\text{GABA}}$ in oocytes expressing $\alpha_1\beta_2\gamma_1$ subunits at 10 (22 ± 2%, $n = 4$) and 100 $\mu$M (64 ± 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 $\mu$M triazolam (A), 10 $\mu$M clotiazepam (B), and 10 $\mu$M midazolam (C). The corresponding mean EC$_{50}$ values were 37 ± 2 $\mu$M (control), 26 ± 4 $\mu$M (triazolam) for $\alpha_1\beta_2\gamma_1$, and 52 ± 7 $\mu$M (control), and 17 ± 3 $\mu$M (triazolam) for $\alpha_1\beta_2\gamma_2S$ (A); 40 ± 8 $\mu$M (control), 25 ± 4 $\mu$M (clotiazepam) for $\alpha_1\beta_2\gamma_1$, and 50 ± 5 $\mu$M (control), and 19 ± 3 $\mu$M (clotiazepam) for $\alpha_1\beta_2\gamma_2S$ (B); and 41 ± 6 $\mu$M (control), 22 ± 2 $\mu$M (midazolam) for $\alpha_1\beta_2\gamma_1$ and 47 ± 11 $\mu$M (control), and 13 ± 6 $\mu$M (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 α1β2γ1 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.

1GABA of α1β2γ1 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 α1β2γ1 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 (EC5–10) by more than 20% with the following order of potency: triazolam > clotiazepam > midazolam > CGS 20625 > CGS 9896 > diazepam. Zolpidem and brezatzenil induced a small enhancement (~13 and 10%, respectively). The effects of DMCM, Cl 218,872, clobazam, flumazenil, Ru 20625, and representatives of other structural classes of ligands of the binding site of GABA_A receptors were also investigated. 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 γ1 Subunit to the Enhancement of 1GABA. 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 α1β2γ1 receptors or could also be explained by effects on α1β2 receptors, the effects of drugs on the latter receptors were also investigated. A comparison of drug effects on α1β2γ1 and α1β2 receptors revealed that the γ1 subunit was essential for enhancement of 1GABA by triazolam, clotiazepam, and midazolam (Table 1 and Fig. 2), because these compounds at 1 μM concentration induced a significant inhibition by ~21 ±

Fig. 6. Modulation of 1GABA by CGS 20625. A, typical 1GABA recordings illustrating concentration-dependent modulation of GABA-elicted chloride currents through α1β2γ1-containing receptors. B, concentration-effect curves for CGS 20625 on α1β2γ1 (□), α1β2γ1 (▲), and α1β2 (■) receptors. EC50 values and corresponding Hill coefficient were the following: 11.2 ± 0.7 μM, nH = 1.8 ± 0.1 (□); 23.7 ± 6.8 μM, nH = 0.9 ± 0.1 (▲); and 4.3 ± 1.2 μM and nH = 1.4 ± 0.2 (■), respectively. Each data point represents mean ± S.E. from at least four oocytes and ≥2 batches. 1GABA at 300 μM (open symbols) were excluded from the fit. C, modulation of the GABA concentration response of α1β2γ1 (left) and α1β2γ2 receptors (right) by 100 μM CGS 20625. The corresponding EC50 values were 39 ± 16 μM (control) and 7 ± 2 μM (CGS 20625) in α1β2γ1 receptors and 56 ± 14 μM (control) and 15 ± 5 μM (CGS 20625) in α1β2γ2 receptors.
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 $\alpha_2\beta$ 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 M) 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 $\gamma_1$-containing receptors (Fig. 3 and Table 2).
The Pyrazolopyridine CGS 20625 Displays the Highest Efficiency in Enhancing GABA-Induced Chloride Flux of α₁β₂γ₁ Subunit Receptors. CGS 20625 was identified as the most efficient compound in terms of maximum enhancement of the GABA-induced chloride currents through α₁β₂γ₁ subunit receptors (Fig. 6). This compound induced a maximum enhancement of 645 ± 55% greater than control, which is approximately 3.75-fold the enhancement achieved with clotiazepam (172 ± 24% greater than control), and more than 7 times the enhancement induced by midazolam (92 ± 8% greater than control) or by triazolam (85 ± 7% above control), respectively (Fig. 3). CGS 20625, however, had a potency for α₁β₂γ₁ receptors (EC₅₀ ≈ 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 α₁β₂γ₁ and α₁β₂γ₂S subunit receptors to an almost similar extent (Fig. 6B). However, a significantly lower efficiency on α₁β₂ receptors revealed an essential role of a γ subunit. CGS 20625 at 300 μM caused less enhancement than at 100 μM in α₁β₂, α₁β₂γ₁, and α₁β₂γ₂S 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 GABAₐ channels (Thompson et al., 2002).

CGS 20625 thus represents a low-potency (EC₅₀ ≈ 20 μM) but high-efficiency modulator of α₁β₂γ₁ and α₁β₂γ₂S subunit-containing receptors (Fig. 6B). This compound was almost not selective for either γ₁ or γ₂ subunits. α₁β₂ Subunit receptors were, however, stimulated to a significantly lesser extent (≈160%) compared with α₁β₂γ₂S subunit receptors (640–730%) (Fig. 6B).

The competitive antagonist flumazenil (1 μM) inhibited IᵣGABA enhancement of α₁β₂γ₂S receptors but failed to affect the enhancement of IᵣGABA through α₁β₂γ₁ receptors by triazolam and clotiazepam (Fig. 7, right). These data suggest that flumazenil exhibits either no or a very low affinity for the GABA-BZ binding site of α₁β₂γ₁ receptors, that flumazenil interacts with a binding site different from that for triazolam and clotiazepam at these receptors. The observation that the first condition is consistent with the observation that the affinity of flumazenil for its binding site was reduced approximately 1000-fold in GABAₐ receptors in which phenylalanine 77 of the γ₂ subunit was mutated to the corresponding residue (isoleucine) of the γ₁ subunit (γ₂F77I; Buhr et al., 1997; Wingrove et al., 2002; Ogris et al., 2004). At higher concentrations, flumazenil displayed properties of a low-affinity agonist on α₁β₂γ₁ receptors (10 μM potentiated IᵣGABA by 22 ± 2% and 100 μM by 64 ± 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 γ₂F77I point mutation (Ogris et al., 2004), as measured by [³H]flunitrazepam binding studies. A low affinity of these compounds for α₁β₂γ₁ receptors could have contributed to their small effects on these receptors observed in the present study. In contrast, replacement of phenylalanine (γF77I) by the corresponding isoleucine of the γ₁ 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 α₁β₂γ₁ compared with α₁β₂γ₁ receptors could be explained by a reduced apparent affinity of these compounds for α₁β₂γ₁ receptors underlying 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 IᵣGABA in α₁β₂γ₁ 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 α₁β₂γ₂S 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 GABAₐ receptors comprising a γ₁ 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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References


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