The GABA_A Receptor $\alpha + \beta$ – Interface: A Novel Target for Subtype Selective Drugs

Joachim Ramerstorfer, Roman Furtmüller, Isabella Sarto-Jackson, Zdravko Varagic, Werner Sieghart, and Margot Ernst

Department of Biochemistry and Molecular Biology, Center for Brain Research, Medical University Vienna, Austria

GABA_A receptors mediate the action of many clinically important drugs interacting with different binding sites. For some potential binding sites, no interacting drugs have yet been identified. Here, we established a steric hindrance procedure for the identification of drugs acting at the extracellular $\alpha 1 + \beta 3$ – interface, which is homologous to the benzodiazepine binding site at the $\alpha 1 + \gamma 2$ – interface. On screening of >100 benzodiazepine site ligands, the anxiolytic pyrazoloquinoline 2-p-methoxyphenylpyrazolo[4,3-c]quinolin-3(5H)-one (CGS 9895) was able to enhance GABA-induced currents at $\alpha 1 \beta 3$ receptors from rat. CGS 9895 acts as an antagonist at the benzodiazepine binding site at nanomolar concentrations, but enhances GABA-induced currents via a different site present at $\alpha 1 \beta 3 \gamma 2$ and $\alpha 1 \beta 3$ receptors. By mutating pocket-forming amino acid residues at the $\alpha 1$ + and the $\beta 3$ – side to cysteines, we demonstrated that covalent labeling of these cysteines by the methanethio-sulfonate ethylamine reagent MTSEA-biotin was able to inhibit the effect of CGS 9895. The inhibition was not caused by a general inactivation of GABA_A receptors, because the GABA-enhancing effect of ROD 188 or the steroid α -tetrahydrodeoxycorticosterone was not influenced by MTSEA-biotin. Other experiments indicated that the CGS 9895 effect was dependent on the α and β subunit types forming the interface. CGS 9895 thus represents the first prototype of drugs mediating benzodiazepine-like modulatory effects via the $\alpha + \beta$ – interface of GABA_A receptors. Since such binding sites are present at $\alpha \beta$, $\alpha \beta \gamma$, and $\alpha \beta \delta$ receptors, such drugs will have a much broader action than benzodiazepines and might become clinically important for the treatment of epilepsy.

Introduction

GABA_A receptors are the major inhibitory transmitter receptors in the brain. They are ligand-gated chloride channels composed of five subunits that can belong to different subunit classes. The existence of six α , three β , three γ , one δ , one ϵ , one π , one θ , and three ρ subunits in the mammalian nervous system generates an enormous diversity of GABA_A receptor subtypes with different subunit composition. The majority of GABA_A receptors, however, are composed of one $\gamma 2$, two α , and two β subunits (Olsen and Sieghart, 2008). GABA_A receptors are the site of action of a variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, neuroactive steroids, anesthetics, and convulsants (Sieghart, 1995). Due to the action of these drugs, it is now clear that GABA_A receptors are modulating anxiety, the excitability of the brain, muscle tone, vigilance, learning, and memory.

Binding sites for several of these drugs have already been identified on these receptors (Olsen and Sieghart, 2008), but a recent modeling study (Ernst et al., 2005) indicated the presence of multiple solvent-accessible pockets within GABA_A receptors that also could function as possible drug binding sites. Simultaneous drug interaction with several binding sites could explain the extremely

complex pharmacology of these receptors. The benzodiazepines are unique among the drugs interacting with GABA_A receptors, because they only can enhance or reduce ongoing GABAergic activity in contrast to other drugs that also can directly open the GABA_A receptor-associated chloride channel at higher concentrations (Sieghart, 1995). The benzodiazepine binding site is located in the extracellular domain of GABA_A receptors, at the $\alpha + \gamma$ – interface (Sigel, 2002; Ernst et al., 2003), whereas the two GABA binding sites of these receptors are located at the two $\beta + \alpha$ – interfaces (Smith and Olsen, 1995). So far, the remaining extracellular $\alpha + \beta$ – and $\gamma + \beta$ – interfaces have not been systematically investigated as possible drug binding sites. Drugs interacting with the $\alpha + \beta$ – interface should be able to modulate $\alpha \beta$, $\alpha \beta \gamma$, and $\alpha \beta \delta$ receptors and should thus exhibit a much broader action than the benzodiazepines. Nevertheless, such drugs might also be able to distinguish between different receptor subtypes depending on the exact α and β subunit type forming their binding site.

Here, we established a systematic investigation of putative binding pockets for the presence of possible drug binding sites. By using the substituted cysteine accessibility method for sterically hindering access of a drug to its binding site, we identified the first compound mediating part of its pharmacological effects via the extracellular $\alpha 1 + \beta 3$ – interface of GABA_A receptors.

Materials and Methods

GABA_A receptor subunits and point mutations. cDNAs of rat GABA_A receptor subunits $\alpha 1$, $\beta 3$, and $\gamma 2$ S were cloned as described previously (Ebert et al., 1996). cDNAs of the rat subunits $\alpha 2$, $\alpha 3$, and $\alpha 5$ were gifts

Received Sept. 24, 2010; revised Nov. 5, 2010; accepted Nov. 5, 2010.

This work was supported by project P19653 of the Austrian Science Fund (to M.E.) and by the FP7 project HEALTH-F4-2008-202088 (Neurocypres; to W.S.). We thank E. Sigel for helpful discussions.

Correspondence should be addressed to Dr. Margot Ernst, Department of Biochemistry and Molecular Biology, Center for Brain Research, Medical University Vienna, Spitalgasse 4, 1090 Vienna, Austria. E-mail: Margot.Ernst@meduniwien.ac.at.

DOI:10.1523/JNEUROSCI.5012-10.2011

Copyright © 2011 the authors 0270-6474/11/310870-08\$15.00/0

from P. Malherbe (Hoffmann La Roche, Basel, Switzerland). The mutated constructs $\alpha 1V211C$ and $\alpha 1S204C$ were a gift from E. Sigel (Institute of Biochemistry and Molecular Medicine, Bern, Switzerland) and were generated as described previously (Tan et al., 2007). For the generation of mutated $\beta 3$ and $\gamma 2$ subunits, these subunits were subcloned into pCDM8 expression vectors (Invitrogen) as described previously (Tretter et al., 1997). Mutated subunits were constructed by PCR amplification using the wild-type subunit as a template. For this, PCR primers were used to construct point mutations within the subunits by the gene splicing by overlap extension technique (Horton et al., 1993). The PCR primers for $\beta 3T60C$ and $\beta 3Q64C$ contained XmaI and XhoI and primers for $\beta 3M115C$ contained PstI and XhoI or XhoI and XbaI restriction sites, which were used to clone the $\beta 3$ or $\gamma 2$ fragments into pCI vectors, respectively (Promega). The mutated subunits were confirmed by sequencing.

Two-electrode voltage clamp. *In vitro* transcription of mRNA was based on the cDNA expression vectors encoding for GABA_A receptor subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 2$, $\beta 3$, and $\gamma 2$ (all from rat) (Ramerstorfer et al., 2010). After linearizing the cDNA vectors with appropriate restriction endonucleases, capped transcripts were produced using the mMACHINE mMACHINE T7 transcription kit (Ambion). The capped transcripts were polyadenylated using yeast poly(A) polymerase (USB) and were diluted and stored in diethylpyrocarbonate-treated water at $-70^{\circ}C$.

The methods for isolating, culturing, injecting, and defolliculating of oocytes were identical to those described by Sigel et al. (1990) and were described previously (Li et al., 2003). Mature female *Xenopus laevis* (Nasco) were anesthetized in a bath of ice-cold 0.17% Tricain (ethyl-m-aminobenzoate; Sigma) before decapitation and removal of the frog's ovary. Stage 5–6 oocytes with the follicle cell layer around them were singled out of the ovary using a platinum wire loop. Oocytes were stored and incubated at $18^{\circ}C$ in modified Barth's medium [88 mM NaCl, 10 mM HEPES-NaOH, pH 7.4, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.34 mM $Ca(NO_3)_2$] that was supplemented with 100 U/ml penicillin and 100 $\mu g/ml$ streptomycin. Oocytes with follicle cell layer still around them were injected with an aqueous solution of mRNA. A total of 2.5 ng of mRNA per oocyte was injected. Subunit ratio was 1:1:5 for $\alpha 1\beta 3\gamma 2$ receptors and 1:1 for $\alpha 1\beta 3$ receptors consisting of wild-type or mutated $\alpha 1$ subunit together with wild-type or mutated $\beta 3$ subunit. After injection of mRNA, oocytes were incubated for at least 24 h for $\alpha 1\beta 3$ receptors and for at least 36 h for $\alpha 1\beta 3\gamma 2$ receptors before the enveloping follicle cell layers were removed. Collagenase-treatment (type IA; Sigma) and mechanically defolliculating of the oocytes were as described previously (Li et al., 2003).

For electrophysiological recordings, oocytes were placed on a nylon grid in a bath of *Xenopus* Ringer solution (XR; containing 90 mM NaCl, 5 mM HEPES-NaOH, pH 7.4, 1 mM $MgCl_2$, 1 mM KCl, and 1 mM $CaCl_2$). The oocytes were constantly washed by a flow of 6 ml/min XR, which could be switched to XR containing GABA and/or drugs. Drugs were diluted into XR from DMSO solutions, resulting in a final concentration of 0.1% DMSO perfusing the oocytes. Drugs were applied for 30 s before the addition of GABA, which was then coapplied with the drugs until a peak response was observed. Between two applications, oocytes were washed in XR for up to 15 min to ensure full recovery from desensitization. For current measurements, the oocytes were impaled with two microelectrodes (2–3 M Ω) that were filled with 2 M KCl. Maximum currents measured in mRNA-injected oocytes were in the microampere range for all subtypes of GABA_A receptors. To test for modulation of GABA-induced currents by drugs, a concentration of GABA that was titrated to trigger 3% of the respective maximum GABA-elicited current of the individual oocyte (EC_3), was applied to the cell with various concentrations of drugs. Such low GABA concentrations are widely used in the literature. At GABA EC_3 , drug effects are larger than at higher GABA concentrations. In addition, GABA EC_3 probably is close to the GABA concentration acting on extrasynaptic receptors, which represent a large part of all GABA_A receptors in the brain (Farrant and Nusser, 2005). In some experiments, however, the effect of drugs was also investigated at a GABA concentration eliciting 20% of the maximal current (GABA EC_{20}) and, in other experiments, GABA concentration–response curves were performed in the absence or presence of a constant and high drug concentration. MTSEA-biotin solution (2 mM) was freshly made in XR con-

Figure 1. Top view onto the extracellular domain of GABA_A receptors. Each subunit features a plus (+) and a minus (−) side. Binding sites for GABA are located at the interfaces formed by the plus side of the β and the minus side of the α subunits. **A**, $\alpha 1\beta 3\gamma 2$ receptors composed of one $\gamma 2$, two $\alpha 1$, and two $\beta 3$ subunits. The binding site for benzodiazepine ligands (Bz) is located at the interface formed by the plus side of the α and the minus side of the γ subunits. The interface of interest (?) is located at the interfaces formed by the plus side of the α and the minus side of the β subunits. **B**, $\alpha 1\beta 3$ receptors composed of two $\alpha 1$ and three $\beta 3$ subunits. Instead of a binding site for benzodiazepine site ligands, a second interface of interest exists, as well as an interface formed by a $\beta +$ and a $\beta -$ side.

taining the respective GABA- EC_3 concentration. Cells were immediately preincubated in MTSEA-biotin solution for 3 min and washed with XR for 5 min. All recordings were performed at room temperature at a holding potential of -60 mV using a Warner OC-725C two-electrode voltage clamp (Warner Instrument) or a CA-1B oocyte clamp or a TEV-200A two-electrode voltage clamp (both from Dagan). Data were digitized, recorded, and measured using a Digidata 1322A data acquisition system (Molecular Devices). Data were analyzed using GraphPad Prism. Data for GABA-dependent dose–response curve were fitted to the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / 1 + 10^{(\text{LogEC}_{50} - X) \cdot nH}$, where EC_{50} is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50% and nH is the Hill coefficient. Data are given as mean $\pm$ SE from at least three oocytes and two oocyte batches. Statistical significance was calculated using unpaired Student's t test or a one-way ANOVA with Bonferroni's post test.

Results

The pyrazoloquinoline CGS 9895 enhances GABA-induced currents in $\alpha 1\beta 3$ receptors

Both the $\alpha + \beta -$ interface and the benzodiazepine binding site at the $\alpha + \gamma -$ interface contain the $\alpha +$ side (Fig. 1A). A binding site at the $\alpha + \beta -$ interface might thus also accommodate at least some of the benzodiazepine site ligands. To identify drugs possibly mediating some of their effects via the $\alpha + \beta -$ interface and to avoid interaction with the benzodiazepine binding site, we used GABA_A receptors composed of $\alpha 1$ and $\beta 3$ subunits only. Such receptors are assumed to be composed of three β and two α subunits (Tretter et al., 1997; Farrar et al., 1999; Baumann et al., 2001) and should thus have two $\beta 3 + \alpha 1 -$ interfaces (GABA binding sites), two $\alpha 1 + \beta 3 -$ interfaces, and one $\beta 3 + \beta 3 -$ interface, but no benzodiazepine binding site (Fig. 1B). In a screening of >100 benzodiazepine site ligands from different structural classes at $10 \mu M$, the anxiolytic pyrazoloquinoline 2-p-methoxyphenylpyrazolo[4,3-c]quinolin-3(5H)-one (CGS 9895; a gift from Novartis, formerly Ciba-Geigy) (Fig. 2A) (Bennett, 1987) was able to strongly enhance GABA-induced currents in recombinant $\alpha 1\beta 3$ receptors expressed in *Xenopus* oocytes. Subsequent concentration–response curves indicated that CGS 9895 started to enhance GABA-induced currents (GABA EC_3) in these receptors at $1 \mu M$. At $10 \mu M$, this compound was able to stimulate this current up to 400%, and at $30 \mu M$ up to

Figure 2. Structure and pharmacology of CGS 9895. **A**, Structure of CGS 9895. **B**, Concentration–effect curves of CGS 9895 on $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ receptors at GABA EC₃ and GABA EC₂₀. At 30 μ M, CGS 9895 enhanced GABA-induced currents (at GABA EC₃) in $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ receptors to $615 \pm 61\%$ ($n = 5$) and $660 \pm 49\%$ ($n = 5$), respectively. These values are not significantly different. At GABA EC₂₀, 30 μ M CGS 9895 stimulated currents in $\alpha 1\beta 3$ ($n = 4$) and $\alpha 1\beta 3\gamma 2$ ($n = 6$) to $262 \pm 5\%$ and $349 \pm 22\%$, respectively. These values are significantly different ($p < 0.05$). CGS 9895 stimulation of $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ receptors at GABA EC₃ or EC₂₀ was significantly different at 3 μ M ($n = 4$, $p < 0.05$), 10 μ M ($n = 4$ –10, $p < 0.001$), and 30 μ M ($n = 4$ –6, $p < 0.01$). **C, D**, GABA concentration–response curves in the absence (■) or presence (□) of 10 μ M CGS 9895. Effects are normalized to the maximum evoked GABA current. CGS 9895 (10 μ M) evokes a left shift of the GABA EC₅₀ value from 11 to 4 μ M ($p < 0.001$) at $\alpha 1\beta 3$ receptors (**C**), and from 73 to 33 μ M ($p < 0.001$) at $\alpha 1\beta 3\gamma 2$ receptors (**D**). The experiments were performed six to eight times in different oocytes.

600% (Fig. 2B). CGS 9895, however, did not directly elicit a chloride current in the absence of GABA.

CGS 9895 is an antagonist at the benzodiazepine binding site

Since this compound in radioligand displacement studies has been demonstrated previously to exhibit a low nanomolar affinity for the benzodiazepine binding site of GABA_A receptors (Yokoyama et al., 1982; Brown and Martin, 1984), we compared its action at $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ receptors. CGS 9895 did not modulate GABA-induced currents at nanomolar concentrations in $\alpha 1\beta 3\gamma 2$ receptors but, at micromolar concentrations, elicited a comparable current enhancement in $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ receptors (Fig. 2B). This effect could be observed at GABA concentrations eliciting 3% (GABA EC₃) or 20% (GABA EC₂₀) of the maximal GABA current in the respective oocytes (Fig. 2B). In addition, 10 μ M CGS 9895 elicited a left shift in the GABA concentration–response curve in $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ receptors (Fig. 2C,D). Since the CGS 9895 effect was much stronger at GABA EC₃ than at GABA EC₂₀, all following experiments were performed at GABA EC₃. CGS 9895 thus seems not to need the benzodiazepine binding site for producing its effect on these GABA_A receptors. This conclusion was confirmed by experiments indicating that the effect of 10 μ M CGS 9895 on $\alpha 1\beta 3\gamma 2$ receptors in contrast to that of 10 μ M diazepam could not be inhibited by a 10 μ M concentration of the benzodiazepine site antagonist Ro15–1788 (Fig. 3A). It is thus possible that CGS 9895 acts as antagonist at the benzodi-

Figure 3. CGS 9895 is an antagonist at the benzodiazepine site and enhances GABA-induced currents via a different site at $\alpha 1\beta 3\gamma 2$ receptors. **A**, Modulation of GABA EC₃ currents of recombinant $\alpha 1\beta 3\gamma 2$ receptors by 10 μ M CGS 9895 (top) or 10 μ M diazepam (bottom) in the absence or presence of 10 μ M Ro 15–1788. The experiments were performed 10 times with comparable results. **B**, Concentration–response curves for diazepam (X), CGS 9895 (■), and diazepam with 50 nM CGS 9895 (□) on $\alpha 1\beta 3\gamma 2$ receptors. The absence of an effect of a single concentration of CGS 9895 at 50 nM is indicated by ○. conc, Concentration. The experiment was performed four times with comparable results.

azepine binding site and stimulates these receptors via a second, as yet unknown, site, which is also present at $\alpha 1\beta 3$ receptors. This conclusion was confirmed by experiments demonstrating that 50 nM CGS 9895, a concentration that completely saturates the benzodiazepine binding site of GABA_A receptors but does not stimulate GABA-induced currents, was able to completely inhibit the effects of diazepam on $\alpha 1\beta 3\gamma 2$ receptors (Fig. 3B). These data are in agreement with previous results indicating that CGS 9895 is an antagonist at the benzodiazepine binding site (Brown and Martin, 1984; Bennett, 1987).

A steric hindrance approach indicates that CGS 9895 mediates its action via the $\alpha + \beta$ – interface

To finally exclude the possibility that CGS 9895 exerts its potentiating action via the $\alpha + \gamma$ – site in $\alpha 1\beta 3\gamma 2$ receptors, the interaction of this drug with the benzodiazepine binding site was inhibited by using the substituted cysteine accessibility method for introducing a steric hindrance into the pocket. For that, several amino acid residues at the $\gamma 2$ – side of the pocket were selected by using our homology model of GABA_A receptors as a guide (Ernst et al., 2003) and individually mutated to cysteines. The effects of these mutations on GABA-induced current and its modulation by diazepam were then investigated in *Xenopus* oocytes expressing these mutated receptors. Introduction of cysteines into binding pockets in many cases impairs the function of receptors, making interpretation of subsequent steric hindrance experiments difficult. Therefore, we selected those point mutations that produced no or small changes in the properties of the receptors for our experiments. The point mutation $\gamma 2$ M130C, although causing an approximately twofold reduction in GABA potency (Fig. 4A, Table 1), did not significantly change the potency and efficacy of diazepam (Fig. 4C) or CGS 9895 (Fig. 4D) to stimulate the mutated receptor. The apparent reduction in GABA-induced currents indicated in Figure 4B was not significant due to the variability of data, possibly caused by different expression levels of receptors in different oocytes. Wild-type and mutated receptors were then exposed to MTSEA-biotin (2 mM) for 2 min, and GABA-elicited currents as well as their potentiation by diazepam or CGS 9895 were measured before and after MTSEA-biotin exposure (Teissière and Czajkowski, 2001). In wild-type $\alpha 1\beta 3\gamma 2$ receptors, MTSEA-biotin had no significant effects on GABA (Figs. 4A,B), diazepam (Fig. 4C), or CGS 9895

Figure 4. The effects of a steric hindrance introduced into $\alpha 1\beta 3\gamma 2$ receptors via $\gamma 2M130C$ on GABA, diazepam, and CGS 9895 concentration–response curves. **A**, GABA effects normalized to the maximum evoked GABA current. **B**, GABA-evoked currents. Due to the variability of the data (expression levels of receptors), the differences observed in the different concentration–response curves are not significant ($n = 3–8$). **C**, Diazepam effects on $\alpha 1\beta 3\gamma 2$ and $\alpha 1\beta 3\gamma 2M130C$ receptors in the absence or presence of MTSEA-biotin. Top, Concentration–response curves of diazepam ($n = 3–5$). MTSEA-biotin caused no significant difference in the diazepam effects on $\alpha 1\beta 3\gamma 2$ receptors, but did cause a significant difference in the diazepam effects on $\alpha 1\beta 3\gamma 2M130C$ receptors at 100 nM, 1 μM , and 10 μM ($p < 0.05$). Bottom, Individual current traces at GABA EC₃ in the absence or presence of 10 μM diazepam under the conditions indicated. **D**, Effects of CGS 9895 on $\alpha 1\beta 3\gamma 2$ and $\alpha 1\beta 3\gamma 2M130C$ receptors in the absence or presence of MTSEA-biotin. Top, Concentration–response curves of CGS 9895 ($n = 3–8$). Bottom, Individual current traces at GABA EC₃ in the absence or presence of 10 μM CGS 9895 under the conditions indicated. □, $\alpha 1\beta 3\gamma 2$ receptors; ◇, $\alpha 1\beta 3\gamma 2$ receptors plus MTSEA-biotin; ■, $\alpha 1\beta 3\gamma 2M130C$ receptors; X, $\alpha 1\beta 3\gamma 2M130C$ receptors labeled with MTSEA-biotin.

(Fig. 4D). Thus, any effect of this reagent on mutated receptors must have been due to covalent labeling of the introduced cysteines. After MTSEA-biotin treatment, the GABA dose–response curve of the mutated receptor was shifted to the left and the potency of GABA for opening the chloride channel was increased (Fig. 4A,B; Table 1), suggesting covalent labeling of the introduced cysteine and possibly indicating that MTSEA-biotin incorporated into the benzodiazepine site at least partially can cause conformational changes comparable to that of benzodiazepines (Teissière and Czajkowski, 2001). MTSEA-biotin nearly eliminated the stimulation of GABA EC₃ by diazepam in the mutated receptor (Fig. 4C), but did not influence the stimulation of this receptor by CGS 9895 (Fig. 4D). Together, these results indicated that CGS 9895, in contrast to diazepam, did not produce its GABA-agonistic effect via the benzodiazepine binding site. In addition, these results demonstrate that the action of a drug at a binding site can be efficiently inhibited by such a steric hindrance approach without influencing the effects of drugs interacting with another binding site.

Table 1. GABA EC₅₀ and Hill slopes (n_H) of various GABA_A receptors in the absence or presence of 2 mM MTSEA-biotin

Receptor	GABA EC ₅₀ (μM)	n_H
$\alpha 1\beta 3$	11 ± 1.3	0.85
$\alpha 1\beta 3$ MTSEA-biotin	12 ± 2.2	0.76
$\alpha 1\beta 3 + 10 \mu M$ CGS 9895	4.2 ± 0.3	0.81
$\alpha 1V211C\beta 3$	4.9 ± 0.5	0.81
$\alpha 1V211C\beta 3$ MTSEA-biotin	12.8 ± 1.9	0.72
$\alpha 1\beta 3Q64C$	17.1 ± 7.3	0.55
$\alpha 1\beta 3Q64C$ MTSEA-biotin	8.6 ± 0.8	0.70
$\alpha 1V211C\beta 3Q64C$	19.6 ± 1.5	0.65
$\alpha 1V211C\beta 3Q64C$ MTSEA-biotin	23.8 ± 5.9	0.61
$\alpha 1\beta 3\gamma 2$	73.4 ± 4.2	1.17
$\alpha 1\beta 3\gamma 2$ MTSEA-biotin	59.1 ± 22.1	1.08
$\alpha 1\beta 3\gamma 2 + 10 \mu M$ CGS 9895	32.5 ± 3.8	1.05
$\alpha 1\beta 3Q64C\gamma 2$	81.9 ± 11	0.94
$\alpha 1\beta 3Q64C\gamma 2$ MTSEA-biotin	98.5 ± 39.5	0.84
$\alpha 1\beta 3\gamma 2M130C$	225.1 ± 41.6	0.84
$\alpha 1\beta 3\gamma 2M130C$ MTSEA-biotin	99.4 ± 49.2	0.99

To investigate whether CGS 9895 is mediating its potentiating action at GABA_A receptors via the $\alpha + \beta$ interface, we applied a similar steric hindrance approach to this interface. For that, several pocket-forming amino acid residues located at the $\beta 3$ side were mutated to cysteines. Receptors containing the mutated $\beta 3$ subunits together with $\alpha 1$ and $\gamma 2$ subunits were expressed in *Xenopus* oocytes and investigated for the effect of the mutation on GABA-induced currents and their stimulation by diazepam or CGS 9895. The mutation $\beta 3Q64C$ did not significantly change the potency of GABA for enhancing GABA-induced currents in $\alpha 1\beta 3Q64C\gamma 2$ receptors (Fig. 5A; Table 1) and also did not cause a significant change in GABA-induced currents (Fig. 5B). The high variability of the currents observed might have been due to differences in the expression levels of receptors in individual oocytes. Whereas this mutation did not significantly reduce the potency and efficacy of diazepam for stimulation of these receptors (Fig. 5C), the effects of CGS 9895 were significantly reduced by the point mutation in $\alpha 1\beta 3Q64C\gamma 2$ receptors (Fig. 5D). On incubation with MTSEA-biotin, GABA-induced currents (Fig. 5A,B), as well as their stimulation by diazepam (Fig. 5C), were not significantly changed, but the effect of CGS 9895 was further reduced (Fig. 5D), suggesting that CGS 9895 might exert its action via the $\alpha + \beta$ interface.

If this is the case, it should be possible to also block the action of CGS 9895 via the $\alpha 1$ subunit. To avoid an interaction of CGS 9895 with both the $\alpha + \beta$ interface and the benzodiazepine binding site located at the $\alpha + \gamma$ interface, we repeated the steric hindrance approach with GABA_A receptors composed of $\alpha 1$ and $\beta 3$ subunits only. As shown in Figure 6A, the GABA-stimulatory effect of CGS 9895 in $\alpha 1\beta 3Q64C$ was not different from that in $\alpha 1\beta 3$ receptors, but covalent modification of the introduced cysteine by MTSEA-biotin was able to reduce this effect by 50%. In contrast, MTSEA-biotin did not significantly change the effects of CGS 9895 in wild-type $\alpha 1\beta 3$ receptors (Fig. 6A). To identify $\alpha 1$ + amino acid residues suitable for this experiment, we mutated several pocket-forming amino acid residues located at the $\alpha 1$ + side to cysteines. The mutation $\alpha 1V211C$ did not significantly change the potency of GABA (data not shown) and did not change the effects of CGS 9895 on GABA-induced currents (Fig. 6B). On covalently labeling the introduced cysteine by MTSEA-biotin, however, the CGS 9895-induced enhancement of GABA currents was reduced by 50% (Fig. 6B). Similarly, modification of position $\alpha 1S204C$ by MTSEA-biotin also reduced CGS 9895 stimulation (data not shown). Finally,

Figure 5. The effects of a steric hindrance introduced into α 1 β 3 γ 2 receptors via β 3Q64C on GABA, diazepam, and CGS 9895 concentration–response curves. **A**, GABA effects normalized to the maximum evoked GABA current. **B**, GABA-evoked currents. Each concentration–response curve was performed four or five times in independent experiments. Due to the variability of data, there was no significant difference in the GABA-evoked currents under the conditions indicated. **C**, Diazepam effects on α 1 β 3 γ 2 and α 1 β 3Q64C γ 2 receptors, the latter in the absence or presence of MTSEA-biotin. Top, Concentration–response curves of diazepam ($n = 4–5$). MTSEA-biotin caused no significant difference in the diazepam effects under the three conditions. Bottom, Individual current traces at GABA EC₃ in the absence or presence of 10 μ M diazepam under the conditions indicated. **D**, Effects of CGS 9895 on α 1 β 3 γ 2 and α 1 β 3Q64C γ 2 receptors, the latter in the absence or presence of MTSEA-biotin. Top, Concentration–response curves of CGS 9895 ($n = 3–10$). There was a significant reduction of the effects of 10 μ M CGS 9895 in the point mutated receptor ($p < 0.05$), which was enhanced in the presence of MTSEA-biotin ($p < 0.001$). Bottom, Individual current traces at GABA EC₃ in the absence or presence of 10 μ M CGS 9895 under the conditions indicated. □, α 1 β 3 γ 2 receptors; ◇, α 1 β 3 γ 2 receptors plus MTSEA-biotin; ■, α 1 β 3Q64C γ 2 receptors; X, α 1 β 3Q64C γ 2 receptors labeled with MTSEA-biotin.

we investigated the effect of CGS 9895 in α 1V211C β 3Q64C receptors, in which cysteines were introduced at the α 1+ as well as at the β 3– side. As shown in Figure 6C, the two introduced cysteines did not significantly influence the GABA-stimulatory effect of CGS 9895, but covalent labeling of cysteines with MTSEA-biotin nearly completely abolished this effect. In contrast, the effects of ROD 188 (Thomet et al., 2000), interacting with an as yet unknown binding site at the GABA_A receptors (Fig. 6D), or of the steroid α -tetrahydrodeoxycorticosterone (data not shown) on α 1 β 3 receptors, were neither changed by the introduced cysteines at the α 1+ or β 3– side, nor by their covalent modification by MTSEA-biotin, suggesting that receptors were not nonspecifically inactivated by MTSEA-biotin. Together, these data strongly suggest that CGS 9895 exerts its action via the extracellular part of the α 1+ β 3– interface.

In a different approach, we considered the possibility that the presence of CGS 9895 inhibits covalent labeling of the introduced cysteines by MTSEA-biotin. However, due to the irreversible nature of the covalent modification by MTSEA-biotin, which overrules the reversible binding of CGS 9895 in a very short time, and

Figure 6. Steric hindrance introduced via β 3Q64C, α 1V211C, or both mutations inhibits the effect of CGS 9895 but not that of ROD 188 on α 1 β 3 receptors. **A**, Concentration–response curves of CGS 9895 on α 1 β 3 (○), α 1 β 3 receptors plus MTSEA-biotin (◇), α 1 β 3Q64C (■), and α 1 β 3Q64C receptors labeled by MTSEA-biotin (X). Each experiment was performed 5–11 times in different oocytes. The reduction of the CGS 9895 effect by MTSEA-biotin in α 1 β 3Q64C receptors was significant at 10 μ M ($p < 0.01$). **B**, Concentration–response curves of CGS 9895 on α 1 β 3 (○), α 1 β 3 receptors plus MTSEA-biotin (◇), α 1V211C β 3 (■), and α 1V211C β 3 receptors labeled by MTSEA-biotin (X). The experiments were performed 5–11 times in different oocytes. The reduction of the CGS 9895 effect by MTSEA-biotin in α 1V211C β 3 receptors was significant at 10 μ M ($p < 0.001$). **C**, Top, Concentration–response curves of CGS 9895 on α 1 β 3 (○), α 1 β 3 receptors plus MTSEA-biotin (◇), α 1V211C β 3Q64C (■), and α 1V211C β 3Q64C receptors labeled by MTSEA-biotin (X). The experiments were performed 5–10 times in different oocytes. The reduction of the CGS 9895 effect by MTSEA-biotin in α 1V211C β 3Q64C receptors was significant at 10 μ M ($p < 0.05$). Bottom, Individual current traces at GABA EC₃ in the absence or presence of 10 μ M CGS 9895 under the conditions indicated. **D**, Top, Concentration–response curves of ROD 188 on α 1 β 3 (○), α 1 β 3Q64C (■), α 1V211C β 3 (■), α 1V211C β 3Q64C (▼), MTSEA-biotin labeled α 1 β 3Q64C (△), MTSEA-biotin labeled α 1V211C β 3 (□), and MTSEA-biotin labeled α 1V211C β 3Q64C (▽) receptors. The experiments were performed three to five times in different oocytes and there were no significant differences between the curves (wild type vs mutated receptors, or mutated receptors vs mutated receptors in the presence of MTSEA-biotin). Bottom, Individual current traces at GABA EC₃ in the absence or presence of 10 μ M ROD 188 under the conditions indicated.

due to simultaneous and partially opposite changes in currents induced by GABA and CGS 9895 during this reaction, such experiments cannot be definitively interpreted.

To investigate whether the effect of CGS 9895 was mediated via the β 3+ β 3– interface that also is present in α 1 β 3 receptors, *Xenopus* oocytes were injected with β 3 subunits only, and the resulting homo-oligomeric receptors were investigated. In agreement with previous results, the ion channel formed by these homo-oligomeric β 3 receptors were open in the absence of GABA and could be modulated by pentobarbital and blocked by picrotoxin (Slany et al., 1995; Woollorton et al., 1997). In contrast, CGS 9895 was not able to change the currents mediated by this channel (data not shown), indicating that either no binding site for this compound can be formed by the β 3+ β 3– interface

in these receptors or that CGS 9895 modulation of this channel via this interface is not possible.

In previous studies (Walters et al., 2000), it was demonstrated that diazepam at concentrations of $>10 \mu\text{M}$ was able to potentiate GABA-induced currents in $\alpha 1\beta 2$ receptors. We demonstrated a similar potentiation of GABA-induced currents by 30–100 μM diazepam on $\alpha 1\beta 3$ receptors. The effects at 100 μM diazepam were similar in $\alpha 1\beta 3$ and $\alpha 1V211C\beta 3Q64C$ receptors but, in contrast to the CGS 9895 effects, could not be significantly inhibited by MTSEA-biotin (data not shown). This is consistent with the suggestion that this low-potency diazepam effect might be caused by an interaction with the transmembrane domain of the receptor (Walters et al., 2000; Baur et al., 2008).

While this work was in progress, evidence was presented indicating that flurazepam, which has been shown to interact with at least two different binding sites in $\alpha 1\beta 2\gamma 2$ receptors (Walters et al., 2000), not only enhanced GABA-induced current via the benzodiazepine binding site, but at concentrations $>10 \mu\text{M}$ also inhibited this current by interacting with the $\alpha 1 + \beta 2$ – interface (Baur et al., 2008). We thus investigated whether flurazepam causes its inhibiting effect at high concentrations via the CGS 9895 binding site at the $\alpha 1 + \beta 3$ – interface. Results indicated that flurazepam at 250 μM was able to inhibit the GABA-stimulatory effect of 10 μM CGS 9895 in $\alpha 1\beta 3$ receptors from $413 \pm 25\%$ to $167 \pm 28\%$ ($n = 4$, $p < 0.001$). In dose-response experiments, flurazepam exhibited no effects in $\alpha 1\beta 3$ receptors up to 1 μM . At higher concentrations, this compound dose dependently inhibited GABA EC₃, indicating that, at this receptor, flurazepam in high concentrations behaves as a negative allosteric modulator (data not shown). This negative modulatory effect of flurazepam, however, could not be inhibited by covalently modifying residue $\beta 3Q64C$, $\alpha 1V211C$, or both by using MTSEA-biotin (data not shown). In addition, this negative modulatory effect at $\alpha 1\beta 3$ receptors, in contrast to the positive modulatory effect of CGS 9895, could not be inhibited by the covalently labeling of residue $\alpha 1S204C$ using MTSEA-biotin (data not shown). The absence of inhibition of the negative allosteric effect of high concentrations of flurazepam by all these covalently modified residues seems to indicate that the negative allosteric effect of flurazepam in $\alpha 1\beta 3$ receptors is not mediated via the extracellular $\alpha 1 + \beta 3$ – interface.

CGS 9895 differentially modulates different receptor subtypes via its binding site at the $\alpha + \beta$ – interface

To investigate a possible receptor subtype selectivity of CGS 9895, receptors containing the $\beta 3$ and different α subunits were investigated. As shown in Figure 7A, CGS 9895 exhibited the highest stimulation of GABA-induced current in receptors composed of $\alpha 1\beta 3$ subunits. In receptors composed of $\alpha 2\beta 3$, $\alpha 3\beta 3$, or $\alpha 5\beta 3$ subunits, the effect of CGS 9895 for stimulating GABA-induced currents was only half of that for $\alpha 1\beta 3$ receptors. Similar results were obtained in receptors composed of $\alpha\beta 3\gamma 2$ subunits. At 10 μM , the effects of CGS 9895 on GABA-induced current in $\alpha 1\beta 3\gamma 2$ receptors was approximately twice as high as that on receptors composed of $\alpha 2\beta 3\gamma 2$, $\alpha 3\beta 3\gamma 2$, or $\alpha 5\beta 3\gamma 2$ subunits (Fig. 7C). Interestingly, however, in contrast to $\alpha 1\beta 3\gamma 2$, $\alpha 2\beta 3\gamma 2$, and $\alpha 5\beta 3\gamma 2$ receptors, which could not be modulated at low CGS 9895 concentrations, receptors composed of $\alpha 3\beta 3\gamma 2$ subunits could be weakly stimulated (up to 130% at 100 nM) at nanomolar concentrations, indicating that CGS 9895 is a weak positive allosteric modulator of $\alpha 3\beta 3\gamma 2$ receptors at these concentrations. Since this effect could be inhibited by covalent labeling of $\gamma 2M130C$ with MTSEA-biotin in $\alpha 3\beta 3\gamma 2M130C$ receptors (Fig. 7D), this weak positive modulatory effect was mediated via the

Figure 7. CGS 9895 effects in GABA_A receptors containing different α or β subunits. **A**, Concentration–response curves of CGS 9895 on $\alpha 1\beta 3$ (■), $\alpha 2\beta 3$ (▲), $\alpha 3\beta 3$ (▼) and $\alpha 5\beta 3$ (●) receptors. The experiments were performed 3–11 times in different oocytes. The effects of 10 μM CGS 9895 were significantly different for $\alpha 1\beta 3$ receptors ($p < 0.01$, one-way ANOVA with Bonferroni's post test). **B**, Concentration–response curves of CGS 9895 on $\alpha 1\beta 1$ (▽), $\alpha 1\beta 2$ (○), $\alpha 1\beta 3$ (■), and $\alpha 1\beta 1S290N$ (▼) receptors. The experiments were performed 4–11 times in different oocytes. The effects of 10 μM CGS 9895 were significantly different for $\alpha 1\beta 1$, but not for the other receptors ($p < 0.001$). **C**, Concentration–response curves of CGS 9895 on $\alpha 1\beta 3\gamma 2$ (■), $\alpha 2\beta 3\gamma 2$ (▲), $\alpha 3\beta 3\gamma 2$ (◆), and $\alpha 5\beta 3\gamma 2$ (▼) receptors. The experiments were performed 3–10 times in different oocytes. The effects of CGS 9895 at 10 and 100 nM were significantly different at $\alpha 3\beta 3\gamma 2$ compared with the other receptors ($p < 0.05$). The effects of 10 μM CGS 9895 were significantly different only between $\alpha 1\beta 3\gamma 2$ and $\alpha 5\beta 3\gamma 2$ receptors ($p < 0.01$, one-way ANOVA with Bonferroni's post test). **D**, Concentration–response curves of CGS 9895 on $\alpha 3\beta 3\gamma 2$ (◆), $\alpha 3\beta 3\gamma 2M130C$ (■), and MTSEA-biotin labeled $\alpha 3\beta 3\gamma 2M130C$ (X) receptors. The effect of 10 and 100 nM CGS 9895 at $\alpha 3\beta 3\gamma 2$ receptors was reduced at $\alpha 3\beta 3\gamma 2M130C$ and MTSEA-biotin labeled $\alpha 3\beta 3\gamma 2M130C$ receptors. The experiments were performed four to six times in different oocytes.

benzodiazepine binding site. These data indicate that CGS 9895 is an antagonist at the benzodiazepine binding site of $\alpha 1\beta 3\gamma 2$, $\alpha 2\beta 3\gamma 2$, or $\alpha 5\beta 3\gamma 2$ receptors, but a weak positive allosteric modulator at the benzodiazepine site of receptors composed of $\alpha 3\beta 3\gamma 2$ subunits.

We also investigated the influence of the type of the β subunit on the CGS 9895 stimulation of GABA-induced current. Results obtained indicated that this compound exhibits a comparable stimulation of GABA-induced currents in $\alpha 1\beta 3$ and $\alpha 1\beta 2$ receptors, but nearly no stimulation in $\alpha 1\beta 1$ receptors (Fig. 7B). The lack of effect of CGS 9895 on $\beta 1$ -containing receptors is similar to that of several other GABA modulators, such as loreclezole, etomidate, and DMCM (Stevenson et al., 1995). Since their β selectivity can be modulated by a point mutation of the amino acid residue 290, which is an Asn in the $\beta 2$ or $\beta 3$ subunit and a Ser in the $\beta 1$ subunit, we investigated whether the mutation $\beta 1S290N$ can enhance the effect of CGS 9895 on $\alpha 1\beta 1$ receptors. As shown in Figure 7B, 10 μM CGS 9895 were able to modulate GABA-induced currents in $\alpha 1\beta 1$ receptors containing the point mutation $\beta 1S290N$ to a similar extent as in $\alpha 1\beta 2$ or $\alpha 1\beta 3$ receptors. Since residue $\beta 1S290N$ is located within TM2 of this subunit and is far away from the putative binding site of CGS 9895 in the extracellular domain (distance of $\beta 3N290$ to $\alpha 1V211C$ or $\beta 3Q64C$ is approximately twice the length of MTSEA-biotin),

these data suggest that, in this case, N290 probably is not involved in direct binding of this compound but in the transduction of its effect on GABA-induced chloride current.

Discussion

Steric hindrance: a novel tool for the identification of drug binding sites

A variety of clinically important drugs exert their effects via GABA_A receptors, but additional drug binding sites might be present at these receptors (Ernst et al., 2005). To develop a tool for identifying possible drug binding sites at GABA_A receptors, we established a steric hindrance approach. In a proof of principle, we introduced a cysteine at the $\gamma 2$ – side ($\gamma 2M130C$) of the benzodiazepine binding pocket and demonstrated that covalently labeling of this cysteine with MTSEA-biotin in $\alpha 1\beta 3\gamma 2M130C$ receptors drastically reduces the ability of diazepam to stimulate GABA-induced ion flux without altering the effects of drugs interacting with other binding sites of these receptors. This was expected because MTSEA-biotin, with its length of 12 Å (Teissière and Czajkowski, 2001), when irreversibly bound to a cysteine located within a pocket, cannot reach into another binding pocket. Due to its length and flexibility, however, this compound cannot be used for exactly localizing the binding site within the pocket.

We then used a similar approach for identifying drugs interacting with the $\alpha 1 + \beta 3$ – interface by introducing cysteines at the $\alpha 1 +$ ($\alpha 1V211C$) and $\beta 3$ – ($\beta 3Q64C$) sides and subsequently investigating whether covalent labeling of these cysteines by MTSEA-biotin could inhibit the effects of the drugs. The pyrazoloquinoline CGS 9895, which previously has been demonstrated to exhibit a high affinity for the benzodiazepine binding site of GABA_A receptors (Yokoyama et al., 1982; Brown and Martin, 1984), was selected for these investigations because this drug was able to enhance GABA-induced currents in receptors containing $\alpha 1$ and $\beta 3$ subunits only. Several lines of evidence indicated that CGS 9895 exerts its GABA-enhancing action in $\alpha 1\beta 3\gamma 2$ receptors via the $\alpha 1 + \beta 3$ – interface. First, this drug at nanomolar concentrations does not modulate GABA-induced currents, but completely prevents the action of diazepam in $\alpha 1\beta 3\gamma 2$ receptors, indicating that it acts as an antagonist at the benzodiazepine binding site (Bennett, 1987). Second, only at micromolar concentrations was CGS 9895 able to enhance GABA-induced currents in $\alpha 1\beta 3\gamma 2$ receptors, but this effect could not be inhibited by the benzodiazepine site antagonist Ro15–1788, again indicating that it is not mediated via the benzodiazepine binding site. Third, CGS 9895 was able to elicit a comparable stimulation of GABA-induced currents in receptors composed of $\alpha 1\beta 3$ or $\alpha 1\beta 3\gamma 2$ subunits, but not in homo-oligomeric receptors composed of $\beta 3$ subunits only. Fourth, the inability of this drug to elicit a current in the absence of GABA and the GABA-enhancing effect of this drug argue against its interaction with the GABA binding sites located at the $\alpha 1$ – $\beta 3 +$ interfaces. Fifth, the GABA-enhancing effect of CGS 9895 could be partially inhibited by a steric hindrance mediated by MTSEA-biotin via cysteines introduced at the $\alpha 1 +$ ($\alpha 1V211C$) and $\beta 3$ – ($\beta 3Q64C$) side, and was even more inhibited when the steric hindrance was introduced via both the $\alpha 1 +$ and the $\beta 3$ – side, finally supporting the conclusion that the respective binding site is located at the extracellular part of the $\alpha 1 + \beta 3$ – interface.

It has been shown previously that some benzodiazepine site ligands can also interact with GABA_A receptors via binding sites different from the classical benzodiazepine site. For instance, Hauser et al. (1997) demonstrated that [³H]flunitrazepam exhibits a high affinity binding site at $\alpha 6\beta 2\gamma 2$ receptors, although at

very high concentrations, this compound was able to displace [³H]Ro15–4513 binding from the respective benzodiazepine binding site. This high-affinity flunitrazepam binding obviously elicited a negative modulatory effect at these receptors. In other experiments, it was demonstrated that the negative allosteric modulator [³H]Ro15–4513 exhibited a high affinity binding to $\alpha 4/6\beta 3\delta$ GABA_A receptors that could be displaced by several other benzodiazepine site ligands (Hancher et al., 2006). Finally, Walters et al. (2000) reported that, in $\alpha 1\beta 2\gamma 2$ receptors, diazepam at GABA EC₃ not only enhanced GABA-induced currents at a nanomolar concentration via the benzodiazepine binding site, but also exhibited an additional stimulation of GABA-induced currents at micromolar concentrations, which, in contrast to the nanomolar component, could not be inhibited by the benzodiazepine antagonist flumazenil and was also present in $\alpha 1\beta 2$ receptors. This site was possibly located within the membrane-spanning region of the receptor because it could be eliminated by introducing three point mutations into this region. Walters et al. (2000) also demonstrated that flurazepam at high micromolar concentrations produced an inhibitory effect at $\alpha 1\beta 2\gamma 2$ receptors and speculated that this might reflect a desensitization of the GABA current. Finally, Baur et al. (2008) provided some evidence that the site mediating the inhibitory action of flurazepam might be located at the extracellular $\alpha 1 + \beta 2$ – interface of GABA_A receptors. Here, we demonstrated that flurazepam could inhibit the GABA-enhancing effect of CGS 9895 in $\alpha 1\beta 3$ receptors. Actually, flurazepam at high micromolar concentrations acted as a negative allosteric modulator at $\alpha 1\beta 3$ receptors, but this effect could not be inhibited by covalently modifying $\alpha 1V211C$, $\alpha 1S204C$, or $\beta 3Q64C$ by MTSEA-biotin, suggesting that this flurazepam effect was mediated via a site different from that of CGS 9895 in $\alpha 1\beta 3$ receptors. This finding and the multiple binding sites possibly present at GABA_A receptors (Ernst et al., 2005) underscore the importance of the present approach, providing a tool for localizing individual drug binding sites in GABA_A receptors.

The anxiolytic actions of CGS 9895 seem to be mediated via the benzodiazepine binding site of GABA_A receptors containing $\alpha 3$ subunits

CGS 9895 has been demonstrated previously to be a potent ligand for the benzodiazepine binding site of GABA_A receptors. *In vivo*, it elicited its anti-anxiety effect at a dose comparable to that of diazepam (Bennett, 1987). The anxiolytic effect of CGS 9895 thus cannot have been elicited via the micromolar effect of this compound at $\alpha 1\beta 3\gamma 2$ receptors. We therefore investigated the effects of CGS 9895 on various other GABA_A receptor subtypes. Results indicated that CGS 9895 at nanomolar concentrations exhibited a weak positive allosteric effect on $\alpha 3\beta 3\gamma 2$ receptors that could be completely inhibited by MTSEA-biotin in $\alpha 3\beta 3\gamma 2M130C$ receptors, suggesting that it was mediated via the benzodiazepine binding site of these receptors. This effect at nanomolar concentrations could not be observed in $\alpha 1\beta 3\gamma 2$, $\alpha 2\beta 3\gamma 2$, or $\alpha 5\beta 3\gamma 2$ receptors, supporting the assumption that the anxiolytic effect of this compound is mediated via $\alpha 3\beta 3\gamma 2$ receptors. The absence of muscle relaxant and sedative, as well as locomotor effects of CGS 9895 (Brown and Martin, 1984; Bennett, 1987), can be explained by the absence of effects at GABA_A receptors containing $\alpha 1$ or $\alpha 5$ subunits at low concentrations. In addition, the observation that CGS 9895 is able to antagonize the anticonvulsant (Brown et al., 1984) or muscle incoordination effects of diazepam in the rotarod test (Bennett, 1987) at low concentrations is consistent with the antagonistic effects of this compound on the action of diazepam on $\alpha 1\beta 3\gamma 2$ receptors. In contrast, CGS

9895 at 100 mg/kg per os caused a 30% protection against the pentylenetetrazole-induced convulsions in the rat (Bennett, 1987). At such concentrations, plasma levels of CGS 9895 between 20 and 50 μ M were reached and thus, this effect could also have been generated via the effects of this compound elicited at micromolar concentrations on $\alpha 1\beta 3\gamma 2$ receptors.

Drugs interacting with the $\alpha + \beta$ – interface are candidates for a novel GABA_A receptor pharmacology

Although most of the actions of CGS 9895 at low drug concentration seem to be mediated via the benzodiazepine binding site of GABA_A receptors, drugs interacting via the extracellular $\alpha 1 + \beta 3$ – interface should exhibit highly interesting properties. Judged by the observation that CGS 9895, even at high concentrations, can only enhance GABA-induced currents but not directly activate these receptors, drugs acting via the $\alpha + \beta$ – interface will only have GABA-modulatory properties, like the benzodiazepines do. In contrast to the benzodiazepines, however, these drugs will interact with receptors composed of $\alpha\beta$, $\alpha\beta\gamma$, and $\alpha\beta\delta$ subunits and should thus exhibit a much broader anticonvulsive action than benzodiazepines. In addition, these drugs will exhibit receptor subtype-selective actions depending on the exact α or β subunit type forming at the binding site and thus dramatically expand the pharmacology of GABA_A receptor subtypes and our ability to modulate specific receptor subtypes. This conclusion is supported by the present results indicating a difference in the ability of CGS 9895 for stimulating GABA-induced currents on $\alpha\beta\gamma$ receptors containing different α or β subunits. CGS 9895 is the first compound unequivocally identified to modulate GABA_A receptors via the extracellular $\alpha 1 + \beta 3$ – interface. Although its potency for interacting with this site is relatively low, this compound now can be used as a screening tool for the identification of other possible candidates interacting with this binding site. This approach is especially important for the identification of possible antagonists at this binding site. At present, in the absence of a direct effect of such compounds at GABA_A receptors, they only can be detected by their inhibition of the effects of CGS 9895. In addition, using the cysteine mutations $\alpha 1V211C$, $\alpha 1S204C$, and $\beta 3Q64C$, and their modification by MTSEA-biotin, compounds that cause a positive or negative modulation of GABA_A receptors via this binding site can now be identified and that might lead to the development of clinically important drugs with a different spectrum of action.

References

- Baumann SW, Baur R, Sigel E (2001) Subunit arrangement of gamma-aminobutyric acid type A receptors. *J Biol Chem* 276:36275–36280.
- Baur R, Tan KR, Lüscher BP, Gonthier A, Goeldner M, Sigel E (2008) Covalent modification of GABA_A receptor isoforms by a diazepam analogue provides evidence for a novel benzodiazepine binding site that prevents modulation by these drugs. *J Neurochem* 106:2353–2363.
- Bennett DA (1987) Pharmacology of the pyrazolo-type compounds: agonist, antagonist and inverse agonist actions. *Physiol Behav* 41:241–245.
- Brown C, Martin I, Jones B, Oakley N (1984) In vivo determination of efficacy of pyrazoloquinolinones at the benzodiazepine receptor. *Eur J Pharmacol* 103:139–143.
- Brown CL, Martin IL (1984) Modification of pyrazoloquinolinone affinity by GABA predicts efficacy at the benzodiazepine receptor. *Eur J Pharmacol* 106:167–173.
- Ebert V, Scholze P, Sieghart W (1996) Extensive heterogeneity of recombinant gamma-aminobutyric acid A receptors expressed in alpha 4 beta 3 gamma 2-transfected human embryonic kidney 293 cells. *Neuropharmacology* 35:1323–1330.
- Ernst M, Brauchart D, Boresch S, Sieghart W (2003) Comparative modeling of GABA_A receptors: limits, insights, future developments. *Neuroscience* 119:933–943.
- Ernst M, Bruckner S, Boresch S, Sieghart W (2005) Comparative models of GABA_A receptor extracellular and transmembrane domains: important insights in pharmacology and function. *Mol Pharmacol* 68:1291–1300.
- Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. *Nat Rev Neurosci* 6:215–229.
- Farrar SJ, Whiting PJ, Bonnert TP, McKernan RM (1999) Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. *J Biol Chem* 274:10100–10104.
- Hanchar HJ, Chutrinopkun P, Meera P, Supavilai P, Sieghart W, Wallner M, Olsen RW (2006) Ethanol potentially and competitively inhibits binding of the alcohol antagonist Ro15–4513 to alpha4/6beta3delta GABA_A receptors. *Proc Natl Acad Sci U S A* 103:8546–8551.
- Hauser CA, Wetzel CH, Berning B, Gerner FM, Rupprecht R (1997) Flunitrazepam has an inverse agonistic effect on recombinant alpha6beta2gamma2-GABA_A receptors via a flunitrazepam-binding site. *J Biol Chem* 272:11723–11727.
- Horton RM, Ho SN, Pullen JK, Hunt HD, Cai Z, Pease LR (1993) Gene splicing by overlap extension. *Methods Enzymol* 217:270–279.
- Li X, Cao H, Zhang C, Furtmueller R, Fuchs K, Huck S, Sieghart W, Deschamps J, Cook JM (2003) Synthesis, in vitro affinity, and efficacy of a bis 8-ethynyl-4H-imidazo[1,5a]-[1,4]benzodiazepine analogue, the first bivalent alpha5 subtype selective BzR/GABA(A) antagonist. *J Med Chem* 46:5567–5570.
- Olsen RW, Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function: update. *Pharmacol Rev* 60:243–260.
- Ramerstorfer J, Furtmüller R, Vogel E, Huck S, Sieghart W (2010) The point mutation gamma2F77I changes the potency and efficacy of benzodiazepine site ligands in different GABA(A) receptor subtypes. *Eur J Pharmacol* 636:18–27.
- Sieghart W (1995) Structure and pharmacology of γ -aminobutyric acid_A receptor subtypes. *Pharmacol Rev* 47:181–234.
- Sigel E (2002) Mapping of the benzodiazepine recognition site on GABA_A receptors. *Curr Top Med Chem* 2:833–839.
- Sigel E, Baur R, Trube G, Möhler H, Malherbe P (1990) The effect of subunit composition of rat brain GABA_A receptors on channel function. *Neuron* 5:703–711.
- Slany A, Zezula J, Tretter V, Sieghart W (1995) Rat beta 3 subunits expressed in human embryonic kidney 293 cells form high affinity [³⁵S]t-butylbicyclopentylphosphorothionate binding sites modulated by several allosteric ligands of gamma-aminobutyric acid type A receptors. *Mol Pharmacol* 48:385–391.
- Smith GB, Olsen RW (1995) Functional domains of GABA_A receptors. *Trends Pharmacol Sci* 16:162–168.
- Stevenson A, Wingrove PB, Whiting PJ, Wafford KA (1995) beta-Carboline gamma-aminobutyric acidA receptor inverse agonists modulate gamma-aminobutyric acid via the loreclezole binding site as well as the benzodiazepine site. *Mol Pharmacol* 48:965–969.
- Tan KR, Gonthier A, Baur R, Ernst M, Goeldner M, Sigel E (2007) Proximity-accelerated chemical coupling reaction in the benzodiazepine-binding site of gamma-aminobutyric acid type A receptors: superposition of different allosteric modulators. *J Biol Chem* 282:26316–26325.
- Teissière JA, Czajkowski C (2001) A (beta)-strand in the (gamma)2 subunit lines the benzodiazepine binding site of the GABA_A receptor: structural rearrangements detected during channel gating. *J Neurosci* 21:4977–4986.
- Thomet U, Baur R, Razet R, Dodd RH, Furtmüller R, Sieghart W, Sigel E (2000) A novel positive allosteric modulator of the GABA(A) receptor: the action of (+)-ROD188. *Br J Pharmacol* 131:843–850.
- Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant GABA_A receptor subtype. *J Neurosci* 17:2728–2737.
- Walters RJ, Hadley SH, Morris KD, Amin J (2000) Benzodiazepines act on GABA_A receptors via two distinct and separable mechanisms. *Nat Neurosci* 3:1274–1281.
- Wooltorton JR, Moss SJ, Smart TG (1997) Pharmacological and physiological characterization of murine homomeric beta3 GABA(A) receptors. *Eur J Neurosci* 9:2225–2235.
- Yokoyama N, Ritter B, Neubert AD (1982) 2-Arylpyrazolo[4,3-c]quinolin-3-ones: novel agonist, partial agonist, and antagonist of benzodiazepines. *J Med Chem* 25:337–339.