Molecular mode of action of CGS 9895 at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors

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Abbreviations GABA, γ-aminobutyric acid; $GABA_A$, γ-aminobutyric type A; CGS 9865, pyrazoloquinoline 2-p-methoxyphenylpyrazolo [4,3-c] quinolin-3(5H)-one

Abstract

$GABA_A$ receptors are the main inhibitory neurotransmitter receptors in the brain and are targets for numerous clinically important drugs such as benzodiazepines, anxiolytics and anesthetics. Previously, CGS 9895 was described as a positive allosteric modulator acting through the α+/β− interface in the extracellular domain of $GABA_A$ receptors. The localization of the binding site was based on a steric hindrance approach, rather than on direct effects of point mutations. In the current study we further characterized modulation by this compound that seems to have six sites of action. We investigated $GABA_A$ receptors expressed in *Xenopus laevis* oocytes using voltage-clamp electrophysiology. We have identified the α_1Y209 residue present at this interface as a key residue for CGS 9895 modulation. Additionally, the interaction between this residue and various structural analogs was characterized, allowing tentative positioning of CGS 9895 versus α_1Y209 (rat sequence). Not all compounds were found to be sensitive to mutations at the α_1Y209 residue. In addition, the interaction of CGS 9895 with flurazepam was characterized. Flurazepam is hypothesized to act at the same subunit interface in the extracellular domain. We also provide evidence that the $GABA_A$ receptor harbors additional modulatory sites for CGS 9895 at each of the subunit interfaces in the trans membrane domain.

Introduction

The major inhibitory receptor in the mammalian central nervous system is the γ-aminobutyric acid type A ($GABA_A$) receptor. This receptor is composed of five homologous subunits, arranged around a central Cl^- selective channel (Macdonald *et al*. 1994). While 19 subunits have been cloned (Macdonald *et al*. 1994; Barnard *et al*. 1998; Olsen *et al*. 2008; Sigel and Steinmann 2012), the majority of $GABA_A$ receptors in the brain consist of α_1, β_2 and γ_2 subunits (Macdonald *et al*. 1994; Rabow *et al*. 1995; Barnard *et al*. 1998; Olsen *et al*. 2008; Sigel and Steinmann 2012). Subunit composition and arrangement both affect the pharmacological properties of $GABA_A$ receptors (Sigel *et al.* 1990; Sieghart 1995; Minier and Sigel 2004). In the following we assume that the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor has the subunit arrangement $\gamma_2\beta_2\alpha_1\beta_2\alpha_1$ and the $\alpha_1\beta_2$ receptor has the subunit arrangement $\beta_2\beta_2\alpha_1\beta_2\alpha_1$ (Baumann *et al*. 2001).

Several clinically and experimentally used drugs exert their action via $GABA_A$ receptors, including benzodiazepines, barbiturates, and the intravenous anesthetics propofol and etomidate (Sieghart 1995; Rudolph *et al.* 1999; Nishikawa *et al*. 2002; Jurd *et al.* 2003; Sieghart and Ernst 2005; Li *et al.* 2006; Yip *et al*. 2013; Middendorp *et al*. 2014). A high affinity benzodiazepine binding site on $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors is located at the $\alpha+/\gamma-$ subunit interface in the extracellular domain (Sigel 2002; Sigel and Lüscher 2011). This site is homologous to the GABA binding sites at $\beta+/\alpha-$ interfaces (Sigel and Buhr 1997; Sigel and Lüscher 2011). In addition to the high affinity binding site for benzodiazepines (site 1), there are other low affinity sites. One of these, site 2, is thought to be located at the $\alpha+/\beta-$ interface in the extracellular domain (Baur *et al*. 2008). Later it has been postulated that the pyrazoloquinoline CGS 9895 acts in a similar location (Ramerstorfer *et al.* 2011).

Interestingly, an ethanol antagonist site at the same subunit interface has been postulated in extrasynaptic δ subunit containing receptors (Wallner *et al.* 2014). Other sites designated as sites 3 (Middendorp *et al*. 2015), are located in the membrane and have been shown to be abolished by combined mutations to isoleucine of the homologous residues α_1S269, β_2N265, and γ_2S280 (Walters *et al.* 2000).

It has been demonstrated that CGS 9895 at nM concentrations is a null modulator at site 1, and at μM concentrations a positive allosteric modulator acting at the α+/β– interface in the extracellular domain (Ramerstorfer *et al.* 2011). The localization of the binding site for CGS 9895 was based on a steric hindrance approach using a 18 Å long reactive molecule rather than on direct interference of point mutations with modulation by this compound. The modulatory action of CGS 9895 is sensitive to the types of α and β subunit isoforms present in the receptor (Ramerstorfer *et al.* 2011). Later, additional CGS 9895 analogues acting either as allosteric modulators or null modulators were described (Varagic *et al*. 2013a; Varagic *et al.* 2013b).

In the present study we were interested to identify point mutations interfering with modulation by CGS 9895. We show that the α_1Y209 residue, specifically that located at the α+/β– interface in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, is crucial for modulation of the receptor by CGS 9895. We also describe the existence of additional modulatory sites for this compound in the trans membrane domain of the $GABA_A$ receptor.

Materials and methods

Construction of mutated receptor subunits

The point mutations α_1Y209Q, α_1Y209C, α_1Y209F, α_1S269I, β_2N265I and γ_2S280I were prepared using the QuikChange™ mutagenesis kit (Stratagene, Agilent Technologies, Basel, Switzerland). The numbering of amino acid residues is based on mature rat sequences.

Expression of $GABA_A$ receptors in Xenopus oocytes

Methods used were essentially as described (Middendorp et al. 2015). Briefly, capped cRNAs were synthesized (Ambion, Austin, TX, USA) from the linearized plasmids with a cytomegalovirus promotor (pCMV vectors) containing the different subunits, respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (United States Biologicals, Cleveland, OH, USA). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, the dried pellet dissolved in water and stored at −80°C. cRNA mixtures were prepared from these stock solutions and stored at −80°C.

Animal experiments were carried out in strict accordance to the Swiss ethical guidelines, and have been approved by the local committee of the Canton Bern Kantonstierarzt, Kantonaler Veterinärdienst Bern (BE85/15). Surgery of female adult *Xenopus laevis* was done under anesthesia (0.2 % tricaine solution). Oocytes were prepared, injected and defolliculated as described previously (Sigel 1987; Sigel and Minier 2005). Oocytes were injected with 50 nL of the cRNA solution containing wild-type or mutated rat α_1, β_2 and γ_2 subunits of the

$GABA_A$ receptors at a concentration of 10 nM:10 nM:50 nM (Boileau *et al*. 2002). Injected oocytes were incubated in modified Barth's solution at 18°C for at least 24 h before the measurements.

Functional characterization of the $GABA_A$ receptors

Currents were measured essentially as described (Middendorp et al. 2015). Briefly, we used a modified two-electrode voltage clamp amplifier Oocyte clamp OC-725 (Warner Instruments, Hamden, CT, USA) in combination with a XY-recorder (90% response time 0.1 s) or digitized at 100 Hz using a PowerLab 2/20 using the computer programs Chart (ADInstruments – Europe, Oxford, England). Electrodes were filled with 3 M KCl and had resistances of 0.5 – 0.8 MΩ. Tests with a model oocyte were performed to ensure linearity in the larger current range. The response was linear up to 15 μA. The holding membrane potential was −80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Na-HEPES (pH 7.4). Concentration response curves for the compounds were fitted with the equation $I(c) = I_{max}/[1 + (EC_{50}/c)^n]$, where c is the concentration of the compound, EC_{50} the concentration eliciting half-maximal current amplitude, I_{max} is the maximal current amplitude, I the current amplitude, and n is the Hill coefficient. Maximal current amplitudes (I_{max}) were obtained from the fits of the concentration-response curves. For all receptors studied, modulation was measured at a GABA concentration eliciting 0.7-1.2 % of the maximal GABA current amplitude. GABA was applied twice alone, and 45 s or 1 min applications in combination with the different compounds. The duration of washout periods was 4 min in between agonist or agonist/drug aplications to prevent receptor desensitization. At the beginning of the experiments, GABA applications were repeated when the elicited current amplitude altered by >5%. Potentiation

was calculated by the following equation: $(I_{Modulator + GABA}/I_{GABA} - 1) * 100\%$. The perfusion solution was applied through a glass capillary with an inner diameter of 1.35 mm, the mouth of which was placed about 0.4 mm from the surface of the oocyte. This allowed fast changes in agonist concentration around the oocyte. The rate of change was estimated 70% in less than 0.5 s (Sigel *et al*. 1990). The perfusion system was cleaned between drug applications by washing with DMSO to avoid contamination. All media contained a final concentration of 0.5 % DMSO (v/v) to ensure drug solubility.

All data are from as a minimum of three different oocytes from at least two different batches of oocytes. Data represent mean ± SEM. An unpaired *t*-test was used to compare two means. One-way analysis of variance (ANOVA) was used for multiple comparisons followed by a Tukey *post hoc* test. ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$.

Results

We were interested in determining amino acid residues important for the modulation by CGS 9895 at $GABA_A$ receptors. The α_1Y209Q mutation has been shown to abolish high affinity binding of Ro 15-1788 and flunitrazepam at the $\alpha+/\gamma-$ at site 1 (Buhr *et al*. 1997). As there are two α_1 subunits, there are two α_1Y209 residues in a $\alpha_1\beta_2\gamma_2$ receptor. We hypothesized that this mutation would possibly be able to interfere with modulators acting at the $\alpha+/\beta-$ interface. Here, we report the α_1Y209 residue as a major determinant of CGS 9895 action.

Effects of different point mutations of α_1Y209 on potentiation by CGS 9895

We examined the consequences of different point mutations of the α_1Y209 residue on the potentiation by CGS 9895 of currents elicited by GABA. Wild-type $\alpha_1\beta_2\gamma_2$ receptors and three mutated receptors α_1Y209Q$\beta_2\gamma_2$, α_1Y209C$\beta_2\gamma_2$ and α_1Y209F$\beta_2\gamma_2$ were studied. These receptors were expressed in *Xenopus* oocytes, and investigated using electrophysiological techniques. Fig. 1a indicates the localization of this residue in the receptor, and Fig. 1b shows the structure of CGS 9895. For illustration, the localization of the mutations is shown in the crystalized homomeric β_3 receptor (Miller and Aricescu 2014), where the β_3 subunits were renamed α_1, β_2 and γ_2. Fig. 2a shows original current traces obtained for wild-type $\alpha_1\beta_2\gamma_2$ (top) and mutated α_1Y209Q$\beta_2\gamma_2$ (bottom) receptors. Fig. 2b summarizes the results. For all receptors studied, modulation was measured at a GABA concentration eliciting 0.7-1.2 % of the maximal GABA current amplitude. It should be noted that the mutation α_1Y209Q reduces the EC_{50} for channel opening by GABA in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors about 2-fold (Buhr *et al*. 1997). Potentiation by 10 μM CGS 9895 in $\alpha_1\beta_2\gamma_2$ receptors amounted to 336 ± 6 % (mean ±

SEM, n = 11), and was greatly diminished in α_1Y209Q$\beta_2\gamma_2$ receptors to 38 ± 9 % (mean ± SEM, n = 4, p <0.0001 Tukey *posthoc* test) and in α_1Y209C$\beta_2\gamma_2$ receptors 88 ± 24 % (mean ± SEM, n = 4, p <0.0001 Tukey *posthoc* test). In α_1Y209F$\beta_2\gamma_2$ receptors potentiation was 309 ± 15 % (mean ± SEM, n = 4, p > 0.05 Tukey *posthoc* test), and did not differ from the potentiation observed in wild-type receptors.

To exclude interaction with the benzodiazepine binding site located at the α+/γ– interface, where CGS 9895 acts as a antagonist (Ramerstorfer *et al*. 2011), we studied the effect of potentiation on dual subunit $\alpha_1\beta_2$ receptors. Fig. 3a illustrates original traces for $\alpha_1\beta_2$ (top) and mutant α_1Y209Qβ_2 (bottom) receptors. Fig. 3b shows the averaged data. In $\alpha_1\beta_2$ receptors, potentiation by 10 μM CGS 9895 amounted to 388 ± 22 % (mean ± SEM, n = 6). In α_1Y209Qβ_2 receptors potentiation was reduced to -4 ± 4 % (mean ± SEM, n = 6, p < 0.0001 Tukey *posthoc* test), and in α_1Y209Cβ_2 receptors to 43 ± 14 % (mean ± SEM, n = 4, p < 0.0001 Tukey *posthoc* test). The α_1Y209Fβ_2 receptor showed a similar potentiation amounting to 465 ± 49 % (mean ± SEM, n = 4, p > 0.05 Tukey *posthoc* test) as compared to $\alpha_1\beta_2$ receptors.

Therefore replacement of the tyrosine in the $\alpha_1$209 residue by glutamine or cysteine leads to abolishment or a reduction of modulation by CGS 9895. A more conservative mutation to phenylalanine did not affect potentiation. Our results suggest that CGS 9895 acts through the α+/β– interface in the extracellular domain.

Positive allosteric modulation of $GABA_A$ receptors by CGS 9895 of $\alpha_1\beta_2\gamma_2$ and α_1Y209Q$\beta_2\gamma_2$ receptors

Fig. 4 shows the concentration-response curves obtained for wild-type $\alpha_1\beta_2\gamma_2$ and α_1Y209Q$\beta_2\gamma_2$ receptors. The $\alpha_1\beta_2\gamma_2$ receptor was characterized by an EC_{50} of 7.3 ± 0.8 μM (mean ± SEM, n = 4), and the α_1Y209Q$\beta_2\gamma_2$ mutant receptor by an EC_{50} of 26 ± 3 μM (mean ± SEM, n = 4). Thus, this mutation increased the EC_{50} for CGS 9895 over 3-fold as compared to wild-type receptors. In addition, maximal potentiation was decreased by the mutation. In $\alpha_1\beta_2\gamma_2$ receptor maximal potentiation amounted to 632 ± 148 % (mean ± SEM, n = 4), and in α_1Y209Q$\beta_2\gamma_2$ mutated receptors 252 ± 59 % (mean ± SEM, n = 4).

Effect of α_1Y209Q mutation on potentiation by different structural analogues of CGS 9895

In order to compare the different analogues, we determined the effect of the mutation at a concentration of the respective compound at the concentration effecting half maximal potentiation. We studied the effect of the above point mutation on the potentiation by the structural analogue LAU 177 (Fig. 5). A former report has indicated that this compound potentiates $\alpha_1\beta_3$ receptors expressed in *Xenopus* oocytes with an EC_{50} of 1.0 ± 0.1 μM (Varagic *et al*. 2013a). Fig. 1 shows the structure of all analogues studied. LAU 177 differs from CGS 9895 by different substituents in positions 4 and 8. Potentiation by 1 μM LAU 177 in $\alpha_1\beta_2\gamma_2$ receptors amounted to 478 ± 37 % (mean ± SEM, n = 4). In the mutated α_1Y209Q$\beta_2\gamma_2$ receptor potentiation amounted to 304 ± 30 % (mean ± SEM, n = 4, p = 0.011 t-test). This indicates that LAU 177 is only partially affected by the mutation. 1 μM of the benzodiazepine antagonist Ro 15-1788 did not affect the degree of potentiation in $\alpha_1\beta_2\gamma_2$

receptors. Thus, we excluded that modulation was through the high affinity benzodiazepine binding site (not shown).

We also examined the effect of the α_1Y209Q mutation on the potentiation by compounds differing from CGS 9895 only in one position, LAU 176, CGS 8216 and CGS 9896 (Fig. 1b). As above, the antagonist Ro 15-1788 excluded an action of all these compounds at the benzodiazepine binding site 1 (not shown). 10 μM of the compound CGS 9896 showed a similar potentiation in mutant α_1Y209Q$\beta_2\gamma_2$ receptors with potentiation amounting to 177 ± 35 % (mean ± SEM, n = 4) as in wild-type $\alpha_1\beta_2\gamma_2$ receptors, with 197 ± 14 % (mean ± SEM, n = 4, $p > 0.05$ *t*-test). In contrast, 4 μM LAU 176 showed a markedly decreased potentiation in α_1Y209Q$\beta_2\gamma_2$ as compared to $\alpha_1\beta_2\gamma_2$ receptors. Potentiation in $\alpha_1\beta_2\gamma_2$ receptors by LAU 176 in was 1607 ± 159 % (mean ± SEM, n = 4) and in the mutated α_1Y209Q$\beta_2\gamma_2$ receptor 249 ± 21 % (mean ± SEM, n = 4, $p = 0.0001$ *t*-test). For 10 μM CGS 8216 potentiation was very small amounting to 56 ± 5 % (mean ± SEM, n = 4) in wild type receptors and to -34 ± 5 % (mean ± SEM, n = 4, $p < 0.0001$ *t*-test) in mutated α_1Y209Q$\beta_2\gamma_2$ receptors.

In wild-type $\alpha_1\beta_1\gamma_2$ receptors, potentiation by compound LAU 176 was dramatically enhanced as compared to LAU 177, whereas it was almost lost for CGS 8216. This observation has been made previously (Varagic *et al*. 2013a). Replacement in position R4 of a -CN substituent of -OMe is beneficial for the pair LAU 176/ LAU 177, whereas replacement of -OMe by a -H in this position in CGS 9895 is detrimental for potentiation.

Effect of α_1Y209 mutations on potentiation by flurazepam

Since flurazepam has been hypothesized to act with low affinity at the same interface as CGS 9895, namely the α+/β− interface at the extracellular domain (Baur *et al*. 2008), we wanted to

see if the α_1Y209Q mutation affects the response to flurazepam. Thus, we studied the effect of the α_1Y209Q mutation in the concentration response curve for flurazepam at $\alpha_1\beta_2\gamma_2$ receptors (Fig. 6). As shown previously the concentration dependence potentiation is bell-shaped in wild-type $\alpha_1\beta_2\gamma_2$ receptors (Baur *et al*. 2008). This bell shape has been interpreted as a positive allosteric modulation through the classical benzodiazepine site (site 1), that is counteracted by occupation of the site at the α+/β– interface (site 2) (Baur *et al*. 2008). In the mutated receptor, the concentration dependence of the potentiation by flurazepam was also bell shaped but was greatly shifted to the right. As expected this mutation interferes with the action of flurazepam at the classical benzodiazepine site. At concentrations above 10 μM this compound produced an increase of the current, passing through a maximum at 75-100 μM. Apparent affinities in wild-type receptors and the mutated receptors through site 1 were 0.4 ± 0.1 μM (mean ± SEM, n = 9) and 37 ± 15 μM (mean ± SEM, n = 4) respectively. Inhibition at high concentration of flurazepam has been interpreted as occupation of site 2 (Baur *et al*. 2008). The point mutation increased the EC_{50} for site 2 from 64 ± 9 μM (mean ± SEM, n = 9) to 147 ± 18 μM (mean ± SEM, n = 4).

Flurazepam antagonizes potentiation by CGS 9895

Next we studied if flurazepam interferes with CGS 9895 action. For this purpose we used 100 μM and 300 μM of flurazepam to counteract modulation by this compound. At these concentrations flurazepam shows reduced or no potentiation response of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Fig. 6) (Baur *et al*. 2008). 10 μM of CGS 9895 potentiated the response of $\alpha_1\beta_2\gamma_2$ receptors by 347 ± 11 % (mean ± SD, n = 3). Subsequently, the same oocytes were exposed to the combination of 10 μM CGS 9895 and 100 μM of flurazepam, and a residual

potentiation amounting to 151 ± 10 % (mean ± SD, n = 3) was observed. In another set of experiments, 10 μM of CGS 9895 potentiated the response of $\alpha_1\beta_2\gamma_2$ receptor by 323 ± 10 % (mean ± SEM, n = 4). When the same oocytes were exposed to the combination of 10 μM CGS 9895 and 300 μM of flurazepam residual potentiation of 18 ± 4 % (mean ± SEM, n = 4) was obtained. An original current trace of the latter experiment is shown in Fig. 7. As shown in Fig. 6 at a concentration of 300 μM, flurazepam alone nearly fails to affect current elicited by GABA. In $\alpha_1\beta_2$ receptors 10 μM of CGS 9895 potentiated the response by 329 ± 37 % (mean ± SEM, n = 4), subsequently the same oocytes were exposed to the combination of 10 μM CGS 9895 and 300 μM of flurazepam where potentiation amounted to 73 ± 19 % (mean ± SEM, n = 4). The above results indicate that the ability of flurazepam to inhibit modulation by CGS 9895 could be mediated via the same site in the extracellular α+/β− interface. Alternatively, both modulatory sites must communicate or the effect of their occupation converges at the GABA binding site or on channel gating.

Possible additional sites of action of CGS 9895

Although our observations suggest involvement of the α_1Y209 residue situated at the extracellular α+/β− interface in the potentiation by CGS 9895, this compound could still act additionally at other sites in the receptor. Previously, we and others have observed that many hydrophobic substances act at the low affinity site for diazepam on $GABA_A$ receptors located at subunit interfaces in the trans membrane region (site 3). We wondered if CGS 9895 would also act at these sites. Triple mutant α_1S269Iβ_2N265Iγ_2S280I receptor has previously been shown to lack low affinity diazepam sites (Walters *et al*. 2000). Each mutation is located at different receptor interfaces. Wild-type receptors, α_1S269I$\beta_2\gamma_2$, $\alpha_1\beta_2$N265Iγ_2, $\alpha_1\beta_2\gamma_2$S280I and

α_1S269Iβ_2N265Iγ_2S280I were expressed and investigated. The mutated receptors are abbreviated here as α_1M, β_2M, γ_2M, and $\alpha_1\beta_2M\gamma_2M$. The potency of GABA for channel gating in all these mutated receptors has previously been described (Maldifassi *et al*. 2016). In order to exclude general gating effects caused by these mutations we showed that potentiation by low concentrations of diazepam and by THDOC were not affected (Maldifassi *et al*. 2016).

First we characterized in each receptor the concentration-response relationship for the modulation by diazepam. As shown in Fig. 8a, in wild-type and all mutated receptor forms nM concentrations of diazepam potentiated GABA elicited currents. In $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_2M$ receptors the first phase of potentiation reached a plateau at approximately 0.3 μM of diazepam. An inhibitory phase was observed at wild-type $\alpha_1\beta_2\gamma_2$ receptors at 3-10 μM diazepam and an additional phase of potentiation for both receptors was evident at concentrations above 10-30 μM. Mutated $\alpha_1M\beta_2\gamma_2$, $\alpha_1\beta_2M\gamma_2$, and $\alpha_1\beta_2M\gamma_2M$ receptors lacked this low affinity potentiation by diazepam. The curve was bell-shaped. At high concentrations, approximately 10 μM and above, diazepam produced a significant inhibition of the response to GABA in $\alpha_1M\beta_2\gamma_2$, $\alpha_1\beta_2M\gamma_2$, and $\alpha_1\beta_2M\gamma_2M$ receptors.

Subsequently, we determined the effect of individual site 3 mutations and the triple mutation on potentiation by 10 μM CGS 9895, 10 μM LAU 177 and 200 μM of diazepam. As shown in Fig. 8b, in $\alpha_1M\beta_2\gamma_2$ receptors potentiation by 10 μM CGS 9895 was increased to 614 ± 123 % (mean ± SEM, n = 5, p = 0.007 Tukey *posthoc* test) as compared to wild-type receptors, where potentiation amounted to 337 ± 6 % (mean ± SEM, n = 11) respectively. Potentiation by CGS 9895 was reduced in the $\alpha_1\beta_2M\gamma_2$ receptor mutant to 115 ± 15 % (mean ± SEM, n = 4, p = 0.007 Tukey *posthoc* test). In $\alpha_1\beta_2\gamma_2M$ receptors potentiation was increased and amounted to 496 ± 117 % (mean ± SEM, n = 4, p = 0.049 Tukey *posthoc* test).

Potentiation of the GABA response in $\alpha_1M\beta_2M\gamma_2M$ receptors was reduced to 89 ± 9 % (mean ± SEM, n = 7, p = 0.004 Tukey *posthoc* test).

The single mutations in the α_1 and in the β_2 subunits altered the degree of potentiation by 10 μM LAU 177. Modulation was also strongly impaired in the triply mutated $\alpha_1M\beta_2M\gamma_2M$ receptors. Potentiation by 10 μM LAU 177 in wild-type $\alpha_1\beta_2\gamma_2$ receptors was 796 ± 129 % (mean ± SEM, n = 6), in the mutated $\alpha_1M\beta_2\gamma_2$ receptor 390 ± 65 % (mean ± SEM, n = 4, p = 0.0416 Tukey *posthoc* test), and in $\alpha_1\beta_2M\gamma_2$ receptors amounted to 0.3 ± 4.4 % (mean ± SEM, n = 4, p = 0.0001 Tukey *posthoc* test). In the triple mutated receptor $\alpha_1M\beta_2M\gamma_2M$ potentiation was 17 ± 12 % (mean ± SEM, n = 4, p = 0.0002 Tukey *posthoc* test).

Potentiation by 200 μM diazepam was affected by all mutations. Potentiation in wild-type receptors amounted to 348 ± 68 % (mean ± SEM, n = 4) and was reduced to 63 ± 13 % (mean ± SEM, n = 4, p = 0.0257 Tukey *posthoc* test) in the $\alpha_1M\beta_2M\gamma_2M$ receptor. For the individually mutated receptors potentiation amounted to 55 ± 17 % in $\alpha_1M\beta_2\gamma_2$ (mean ± SEM, n = 4, p = 0.036 Tukey *posthoc* test), -52 ± 16 % in $\alpha_1\beta_2N265I\gamma_2$ (mean ± SEM, n = 4, p = 0.016 Tukey *posthoc* test) and to 974 ± 175 % in $\alpha_1\beta_2\gamma_2M$ receptor (mean ± SEM, n = 4, p = 0.0012 Tukey *posthoc* test). In this case, the α_1M mutation may convert CGS 9895 into a negative allosteric modulator.

The above data suggest that additional modulatory sites for CGS 9895 are present at the α+/β–, β+/α–, γ+/β–, and α+/γ– subunit interfaces located in the trans membrane domain of $GABA_A$ receptors. On the other hand, the structural analog LAU 177 may harbor modulatory sites at the α+/γ– and β+/α– subunit interfaces. $GABA_A$ receptors harbor modulatory sites 3

for diazepam at α+/β–, β+/α–, γ+/β–, and α+/γ– subunit interfaces in the trans membrane domain.

Discussion

The pyrazoloquinoline CGS 9895 at nM concentrations was described as an antagonist at the benzodiazepine high affinity site, but at µM concentrations enhanced GABA induced currents in $\alpha_1\beta_3$ and $\alpha_1\beta_3\gamma_2$ receptors (Ramerstorfer *et al*. 2011). Using a steric hindrance approach together with a homology model of $GABA_A$ receptors (Ernst *et al.* 2005), several possible pocket-forming amino acids at the α+/β– subunit interface were mutated to cysteine. Modification with MTSEA-biotin of the mutated residues α_1S204C, α_1V211C and $\beta_3$64C considerably reduced modulation by CGS 9895, indicating that this compound acts within 18 Å of the mutated residues (Ramerstorfer *et al*. 2011). Because of the length and flexibility of MTSEA-biotin, specific amino acids in contact with CGS 9895 were not identified (Ramerstorfer *et al*. 2011). Our results indicate that the α_1Y209 residue located at the α+/β– interface is important for CGS 9895 action and additionally suggest binding sites at all five subunit interfaces in the trans membrane region.

As the $\alpha_1\beta_2\gamma_2$ receptor contains two α_1 subunits, there are two α_1Y209 residues in the receptor. One residue is located at the α+/γ– interface in the extracellular domain of the receptor (site 1), and is part of the diazepam-binding pocket (Amin *et al.* 1997; Buhr *et al*. 1997). The other residue is located at the extracellular part of the α+/β– interface homologous to the residue located at site 1. Mutation to Q of the α_1Y209 residue leads to loss of Ro15-1788 and flunitrazepam binding at the high affinity diazepam site and reduces the EC_{50} for channel opening by GABA about 2-fold (Buhr *et al.* 1997). We studied if the mutation of this

residue might also interfere with the action of modulators supposedly acting at the extracellular α+/β– interface.

We found that mutations in the α_1Y209 residue affected the ability of CGS 9895 to potentiate GABA elicited currents at $GABA_A$ receptors. Replacement of tyrosine in the $\alpha_1$209 residue for the non-aromatic amino acids glutamine or cysteine, led to a dramatic reduction on the action of CGS 9895. When the same tyrosine residue was replaced with phenylalanine, leading to the removal of the hydroxyl group in the benzene ring, potentiation remained almost unaffected. Thus, the aromatic ring in the α_1Y209 residue is essential for the interaction of the receptor with CGS 9895. The α_1Y209Q mutation led to an increase of the EC_{50} for CGS 9895, and a decrease in the maximal potentiation obtained. Similar observations were made in $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ receptors, indicating that indeed the residue at the α+/β– subunit interface is important. It should be noted that in case a point mutation affects modulation by a compound, the corresponding residue can be involved in the formation of the binding pocket, or alternatively it may affect the allosteric transduction required for modulation. At least the ability of GABA to gate the channel is nearly unaffected by the α_1Y209Q mutation (Buhr *et al*. 1997).

Structural analogs of CGS 9895 were investigated for their sensitivity to mutations in the α_1Y209 residue. All compounds investigated contained a common pyrazoloquinolinone nucleus, with different substituents in R4 and R8 (Fig. 1b). EC_{50} and efficacy (10 μM) of the compounds in $\alpha_1\beta_3$ receptors have been described elsewhere (Varagic *et al*. 2013a). CGS 9895 and LAU 176, are both sensitive to the studied mutation, and have both a -OMe group at position R4, but they differ in the R8 position, where the first has a -H and the latter a -OMe group. Potentiation by CGS 9896 is not significantly affected and that by LAU 177 partially by the α_1Y209Q mutation. CGS 9896 has a -Cl at position R4, and at R8 a hydrogen atom.

LAU 177 has a -CN group at the position R4, and a -OMe group at R8. CGS 8216, which contains -H in both positions was the weakest modulator of GABA currents in $\alpha_1\beta_2\gamma_2$ receptors. A similar result was obtained previously using $\alpha_1\beta_3$ receptors (Varagic *et al*. 2013a). From our observations we conclude that the methoxy group at the R4 may be responsible for the sensitivity of these compounds to the α_1Y209Q mutation and might be apposed to this residue.

Previously, a low affinity site for flurazepam has been postulated at the α+/β– subunit interface. We were interested if flurazepam and CGS 9895 interacted at the same site. We found a) that the mutation α_1Y209Q not only affects modulation by CGS 9895 but also affects the affinity of flurazepam to site 2 and b) that 300 µM flurazepam almost completely prevented modulation by CGS 9895. These results could indicate that flurazepam and CGS 9895 may share a site of action at the α+/β– extracellular interface. Different conclusions have been reached earlier. High concentrations of flurazepam were described to partially prevent the action of CGS 9895 (10 µM) in $\alpha_1\beta_3$ $GABA_A$ receptors, and to inhibit GABA mediated currents (Ramerstorfer *et al*. 2011). Since this negative modulatory effect could not be abolished by modification with MTSEA-biotin of α_1S204C, α_1V211C and $\beta_3$64C, the authors concluded that flurazepam effects must be mediated through a different site (Ramerstorfer *et al*. 2011).

The five-subunit interfaces of $GABA_A$ receptors harbor modulatory sites for high concentrations of diazepam (sites 3). We also investigated if CGS 9895 is affected by mutations in these sites. Potentiation by CGS 9895 was reduced in the triple mutant receptor and by the β_2N265I mutation, and increased by the mutations α_1S269I and γ_2S280I. We concluded that the five-subunit interfaces of $GABA_A$ receptors bear modulatory sites for CGS 9895. The fact the β_1S290N mutation leads to loss of stimulation by CGS 9895 in $\alpha_1\beta_1$

receptors (Ramerstorfer *et al*. 2011) were previously interpreted as allosteric effect. The structural analog LAU 177 was also sensitive to some of the site 3 mutations. Potentiation was reduced in the triple mutant receptor, as by the α_1S269I and β_2N265I mutations, however it was not affected by the γ_2S280I mutation. Therefore only the β+/α–, α+/β–, and α+/γ– $GABA_A$ receptor subunit interfaces have modulatory sites for this compound.

In summary, we show that α_1Y209 at the α+/β– interface in the extracellular domain is a key residue for CGS 9895 action in $GABA_A$ receptors and that it may be in contact with the –OMe substituent of this compound. Additionally, the $GABA_A$ receptor harbors modulatory sites for this compound at subunit interfaces in the membrane.

Acknowledgements and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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Legends to figures

Fig. 1 (a) Model structure of the $GABA_A$ receptor transmembrane domain. The receptor is composed of two α (yellow), two β (blue), and one γ (red) subunits; structures are shown in ribbon representation. Shown in orange are the αY209 residues located at the extracellular $\alpha+/\beta-$ and $\alpha+/\gamma-$ interfaces. The model structure depicts the crystalized homomeric β_3 $GABA_A$ receptor (PDB structure 4COF) (Miller and Aricescu 2014). Some of the β_3 subunits were renamed α_1, β_2 and γ_2. (b) Chemical structures of the compounds studied. Right: The investigated compounds differed in their substituents at position R4 and R8. Left: Common pyrazoloquinolinone nucleus.

Fig. 2 Potentiation of wild-type $\alpha_1\beta_2\gamma_2$ and mutated α_1Y209Q$\beta_2\gamma_2$, α_1Y209C$\beta_2\gamma_2$, α_1Y209F$\beta_2\gamma_2$ receptors by 10 µM CGS 9895. (a) Original current traces showing the potentiation of the GABA elicited response by 10 µM CGS 9895 in wild-type (top) and mutant α_1Y209Q$\beta_2\gamma_2$ (bottom) receptor. Receptors were expressed in *Xenopus* oocytes and electrophysiological experiments were performed. The holding membrane potential was −80 mV. GABA at a concentration eliciting 0.7-1.2 % of the maximal current amplitude (single bars) was applied twice alone and then in combination with 10 µM CGS 9895. The α_1Y209Q mutation strongly decreased allosteric modulation. (b) Summary of the results obtained in wild-type, and mutant α_1Y209Q$\beta_2\gamma_2$, α_1Y209C$\beta_2\gamma_2$, and α_1Y209F$\beta_2\gamma_2$ receptors. Experiments were performed four to eleven times with different oocytes from at least three different batches; data are expressed as mean ± SEM.

Fig. 3 Potentiation of $\alpha_1\beta_2$ and mutated α_1Y209Qβ_2, α_1Y209Cβ_2, α_1Y209Fβ_2 receptors by 10 µM CGS 9895. (a) Original current traces showing the potentiation of the GABA elicited response by 10 µM CGS 9895 in oocytes expressing $\alpha_1\beta_2$ (top) and mutant α_1Y209Qβ_2 (bottom) receptors. GABA at a concentration eliciting 0.7-1.2 % of the maximal current amplitude (single bars) was applied twice alone and then in combination with 10 µM CGS 9895. (b) Summary of the results obtained in $\alpha_1\beta_2$, and mutant α_1Y209Qβ_2, α_1Y209Cβ_2, α_1Y209Fβ_2 receptors. The holding membrane potential was −80

mV. Experiments were performed four to six times in different oocytes from at least three different batches; data are expressed as mean ± SEM.

Fig. 4 Concentration dependence of the positive allosteric modulation by CGS 9895 in oocytes expressing wild-type (closed circle) and α_1Y209Q$\beta_2\gamma_2$ (closed square) receptors. Data are shown as mean ± SEM, n = 4.

Fig. 5 Mutations at the α_1Y209 residue affect potentiation by different structural analogues of CGS 9895. Potentiation of the GABA elicited response by 1 μM LAU 177, 4 μM LAU 176, 10 μM CGS 8216 and 10 μM CGS 9896 in wild-type and mutant α_1Y209Q$\beta_2\gamma_2$ receptors. Experiments were performed four to six times using different oocytes; data are expressed as mean ± SEM.

Fig. 6 Concentration-response curves obtained of flurazepam in wild-type receptors (closed circle), and mutant α_1Y209Q$\beta_2\gamma_2$ receptors (closed square). Experiments were carried out 4-9 times, with three different batches of oocytes. Results are expressed as mean ± SEM.

Fig. 7 Original current traces showing inhibition of CGS 9895 induced $GABA_A$ receptor potentiation by flurazepam (FZM). Wild-type receptors were expressed in *Xenopus* oocytes. GABA was applied two times, followed by co-application of the same concentration of GABA with 10 μM CGS 9895, and finally combined application of GABA with 10 μM CGS 9895 and 300 μM flurazepam. The holding membrane potential was −80 mV. Experiments were carried out 4 times with three different batches of oocytes, with a similar outcome.

Fig. 8 (a) Concentration dependence of the positive allosteric modulation by diazepam in oocytes expressing different receptors: $\alpha_1\beta_2\gamma_2$ (closed circle), α_1M$\beta_2\gamma_2$ (inverted triangle), $\alpha_1\beta_2$Mγ_2 (square), $\alpha_1\beta_2\gamma_2$M (triangle), and α_1Mβ_2Mγ_2M (gray circle) receptors. Data is represented as mean ± SEM, n = 4-5. (b) Summary of the results obtained by 10 μM CGS 9895, 10 μM LAU 177, and 200 μM diazepam

in wild-type, single mutant $\alpha_1M\beta_{2\gamma2}$, $\alpha_1\beta_2M\gamma_2$, $\alpha_1\beta_{2\gamma2}M$ and triple mutant $\alpha_1M\beta_2M\gamma_2M$ receptors. The bars indicate mean $\pm$ SEM, n = 4-5.