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Subunit mutations affect ethanol actions on $GABA_A$ receptors expressed in *Xenopus* oocytes

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1 Mutations of specific amino acids were introduced in transmembrane domains (TM) of $GABA_A$ receptor α_2, β_1 and γ_{2L} subunits. The effects of these mutations on the action of ethanol were studied using the *Xenopus* oocyte expression system and two-electrode voltage-clamp recording techniques.

2 Mutant α_2 subunits containing S270I (TM2) or A291W (TM3) made the receptor more sensitive to GABA, as compared to wild-type $\alpha_2\beta_1\gamma_{2L}$ receptor. The mutation S265I (TM2) of β_1 and S280I (TM2) or S301W (TM3) in γ_{2L} subunits did not alter apparent affinity of the receptor for GABA. M286W (TM3) in the β_1 subunit resulted in a receptor that was tonically open.

3 Using an EC_5 concentration of GABA, the function of the wild-type receptor with $\alpha_2\beta_1\gamma_{2L}$ subunits was potentiated by ethanol (50–200 mM). The mutations in TM2 or TM3 of the α_2 subunit diminished the potentiation by ethanol. The action of ethanol was also eliminated with a mutation in the TM2 site of the β_1 subunit. Ethanol produced significant inhibition of GABA responses in receptors containing the combination of α_2 and β_1 TM2 mutants with a wild-type γ_{2L} subunit. A small but significant reduction in the potentiation by ethanol was observed with γ_{2L} TM2 and/or TM3 mutants.

4 From these results, we suggest that in heteromeric $GABA_A$ receptors composed of the α, β and γ subunits, ethanol may bind in a cavity formed by TM2 and TM3, and that binding to the α or β subunit may be more critical than the γ subunit.

Keywords: $GABA_A$ receptor; mutation; *Xenopus* oocytes; ethanol

Abbreviations: GABA, γ-aminobutyric acid; TM, transmembrane domains

Introduction

Ethanol is one of the oldest and most widely consumed drugs, and has many behavioural effects, some of which are shared with sedative, hypnotic and anaesthetic agents. It is likely that sites for ethanol's action in brain include several neurotransmitter receptors and ion channels (Deitrich *et al*., 1989; Harris *et al*., 1995). One ligand-gated ion channel of interest as a target for ethanol, as well as sedative, hypnotic and anaesthetic drugs, is the γ-aminobutyric acid type A ($GABA_A$) receptor. Recent studies using techniques such as mutagenesis and heterologous expression systems have made it possible to investigate the cellular and molecular mechanisms of the action of ethanol on $GABA_A$ receptors (Mihic & Harris, 1996).

The $GABA_A$ receptor/chloride channel complex is the major inhibitory neurotransmitter receptor in the mammalian brain. It is a member of the ligand-gated ion channel superfamily, which includes glycine, $GABA_C$, 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) and nicotinic acetylcholine (nACh) receptors (Ortells & Lunt, 1995). Five classes of $GABA_A$ receptor subunits (α_{1-6}, β_{1-4}, γ_{1-4}, δ, ε) have been cloned to date (Whiting *et al*., 1992). Because $GABA_A$ receptors expressed *in vitro* without the γ subunit are sensitive to general anaesthetics and n-alcohols (Levitan *et al*., 1988; Pritchett *et al*., 1989; Harrison *et al*., 1993; Mihic *et al*., 1994), the α and β subunits are likely target proteins for anaesthetic agents. Recent studies demonstrated that mutation of specific amino acids in the transmembrane domains (TM) of the α or β subunit of $GABA_A$ receptor can eliminate the action of ethanol, enflurane and isoflurane (Mihic *et al*., 1997; Krasowski *et al*., 1998) without abolishing the response to GABA. These results suggest that the receptors might have specific regions and conformations for the action of alcohols and anaesthetics.

However, only α and β subunits were used in our previous mutation studies. Most $GABA_A$ receptors in brain are composed of α, β and γ subunits with the consensus stoichiometry being $\alpha^2\beta^2\gamma^1$ (Chang *et al*., 1996; Tretter *et al*., 1997), and it was demonstrated that low concentrations of ethanol appear to require the presence of a γ subunit (Wafford *et al*., 1991; Wafford & Whiting, 1992; Harris *et al*., 1997). Therefore, these findings raise several questions: how do the mutations in α or β subunits affect the action of alcohols when $GABA_A$ receptors are composed of α, β and γ subunits? Are there any effects of mutations in the corresponding regions of the γ subunit on the action of alcohols? Mihic *et al*. (1997) tested only a single, high concentration of alcohol: can mutations in α, β or γ subunits differentially affect the action of low and high concentrations of alcohols?

To investigate the importance of the γ subunit, we expressed mutant α_2 or β_1 subunits together with the γ_{2L} subunit in *Xenopus* oocytes and studied effects of a range of concentrations of ethanol. Moreover, we prepared mutant γ_{2L} subunits and investigated the effect of these mutations on actions of ethanol. Previous studies of chimeric and mutant receptors identified one amino acid residue in TM2 and one in TM3 that account for the difference in ethanol action on GABA ρ_1

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($GABA_C$) receptor function (inhibition) as compared to glycine receptors (enhancement) (Mihic *et al*., 1997). The critical residue in TM2 is serine in glycine α_1, $GABA_A$ receptor α_2, α_1, β_1 and γ_2 subunits, but is isoleucine in the GABA ρ_1 receptor subunit (Figure 1). The TM3 residue is alanine in glycine α_1, $GABA_A$ receptor α_2, β_1 and γ_2 subunits, but tryptophan in the GABA ρ_1 receptor subunit (Figure 1). Because mutation of the TM2 serine to isoleucine markedly inhibits the action of ethanol on receptors composed of glycine α_1 or $GABA_A$ receptor α_1 and β_1 subunits (Mihic *et al*., 1997), we also mutated the homologous serine residue in TM2 of $GABA_A$ receptor γ_{2L} subunit to isoleucine for the present studies. Similarly, mutation of alanine to tryptophan in $GABA_A$ receptor α_2 subunit prevents action of ethanol and therefore the homologous serine residue in the γ_{2L} subunit was mutated to tryptophan for our current studies (Figure 1).

Methods

Adult *Xenopus laevis* female frogs were obtained from Xenopus I (Ann Arbor, MI, U.S.A.); GABA from Research Biochemicals International (Natick, MA, U.S.A.); dimethylsulphoxide, collagenase type 1A, picrotoxin and flunitrazepam from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Ethanol from Aaper Alcohol and Chemical Co. (Shelbyville, KY, U.S.A.). All other chemicals used were of reagent grade.

Mutations in cDNAs of human $GABA_A$ receptor α_2 (S270I, A291W) and β_1 (S265I, M286W) subunits were described previously (Krasowski *et al*., 1998). The S280I and S301W mutations in the γ_{2L} subunits were introduced by use of the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The cDNAs of wild-type or mutant receptor subunits were subcloned into the pCIS2 or modified (Wick *et al*., 1998) pBK-CMV (Stratagene) vectors. All point mutations were verified by double-stranded DNA sequencing.

Xenopus oocytes were isolated and injected with cDNAs (1.5 ng per 30 nl), and two-electrode voltage-clamp recordings were performed as described previously (Mihic *et al*., 1994; Harris *et al*., 1997). GABA was applied for 20–30 s and the maximum (peak) current was used as a measure of drug response. For GABA concentration-response curves, we applied 0.03 μM–1 mM GABA solutions for a single oocyte with an interval of 5 min, or 15 min when desensitization was observed. We tested the capacity of ethanol to enhance the effect of administration of GABA concentration that produced

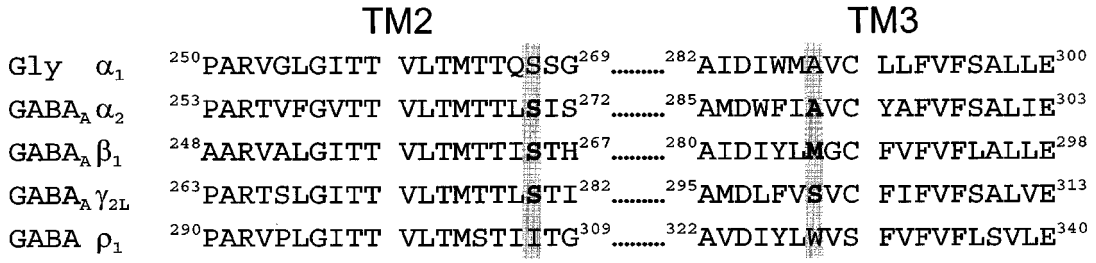

Figure 1 Amino acid sequence alignment of TM2 and TM3 from human glycine α_1, $GABA_A$ α_2, β_1, γ_{2L}, and GABA ρ_1 receptor subunits. The amino acids investigated in the present study are Ser270 and Ala291 in $GABA_A$ receptor α_2, Ser265 and Met286 in β_1 and Ser280 and Ser301 in γ_{2L} subunits (shown as bold letters). The amino acids in TM2 were all mutated to Ile and those in TM3 to Trp (see Methods).

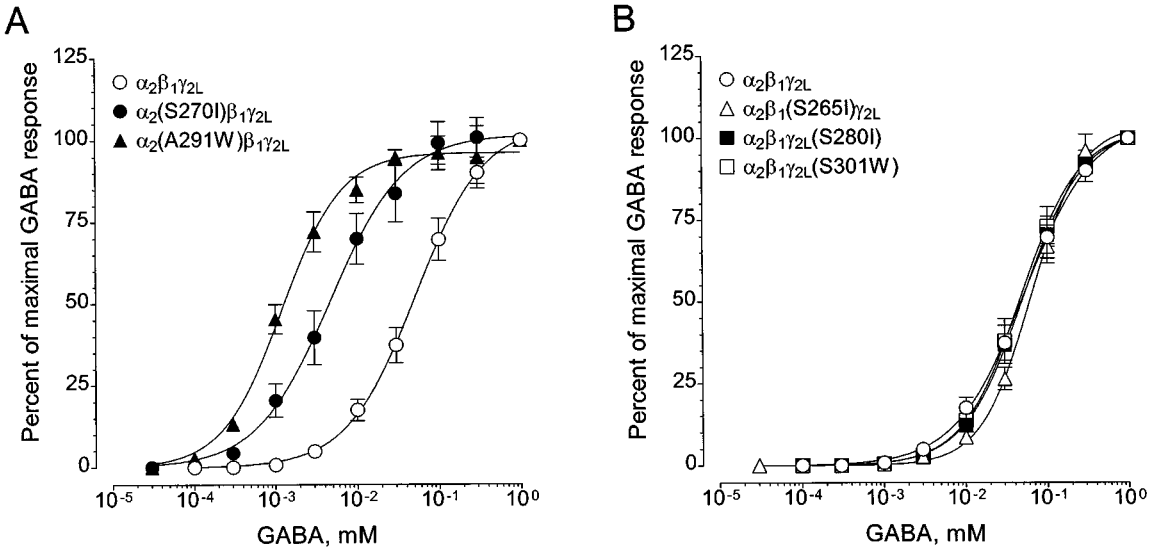

Figure 2 GABA concentration-response curves for wild-type and mutant $GABA_A$ receptors expressed in *Xenopus* oocytes. The curves for mutant α_2(S270I)$\beta_1\gamma_{2L}$ and α_2(A291W)$\beta_1\gamma_{2L}$ are shown in (A) and mutant $\alpha_2\beta_1$(S265I)γ_{2L}, $\alpha_2\beta_1\gamma_{2L}$(S280I) and $\alpha_2\beta_1\gamma_{2L}$(S301W) are in (B) The curve for wild-type $\alpha_2\beta_1\gamma_{2L}$ are shown in both (A) and (B). Nonlinear regression analysis of the curves was performed as described in 'Methods', and the results shown in Table 1. Values are presented as mean ± s.e.mean from 4–6 oocytes. In some cases, the error bars are smaller than the points.

5% of the maximal effect (EC_5) of GABA, because there is a dependence on the GABA concentration with greater potentiation by ethanol being seen at the lower GABA concentrations (Mihic *et al*., 1994). This EC_5 was determined individually for each oocyte. We used 1 mM GABA to produce a maximal current. Oocytes were perfused with ethanol for 2 min before coapplication of GABA, to allow for complete equilibration of the oocytes with ethanol. In all cases, a 15–20 min washout period was allowed following application of the ethanol/GABA solutions. The solutions were freshly prepared immediately before use. Each data point represents a mean from 3–38 oocytes obtained from at least two different frogs. Across all the potentiation experiments for wild-type and mutant receptors reported here, the actual percentage of maximal GABA response for the test concentrations used here were: $\alpha_2\beta_1\gamma_{2L}$ wild-type (4.9±0.1%, total 58 experiments), α_2(S270I)$\beta_1\gamma_{2L}$ (5.0±0.2%, 20 experiments), α_2(A291W)$\beta_1\gamma_{2L}$ (5.0±0.1%, 24 experiments), $\alpha_2\beta_1$(S265I)γ_{2L} (5.1±0.2%, 14 experiments), α_2(S270I)β_1(S265I)γ_{2L} (5.0±0.2%, 9 experiments), $\alpha_2\beta_1\gamma_{2L}$(S280I) (5.0±0.3%, 14 experiments), $\alpha_2\beta_1\gamma_{2L}$(S301W) (5.1±0.1%, 15 experiments) and $\alpha_2\beta_1\gamma_{2L}$(S280I/S301W) (5.0±0.1%, 22 experiments).

All values are presented throughout as mean±standard error of the mean (s.e.mean). Statistical analyses were carried out by one-way analysis of variance (ANOVA) with Bonferroni's comparison *post hoc* test, paired or unpaired, two-tailed *t*-test using GraphPad Prism software (San Diego, CA, U.S.A.). Curve fitting and estimation of EC_{50} values for concentration-response curves were also performed using this software.

Table 1 GABA EC_{50}s, Hill slopes and maximal responses for wild-type and mutant GABA$_A$ receptors expressed in *Xenopus* oocytes

Subunit composition	*GABA EC_{50} (μM)*	*Hill slope*	*Response to 1 mM GABA* nA	*% of maximal*
$\alpha_2\beta_1\gamma_{2L}$	61±17	1.1±0.1	6995±1730	94±1
α_2(S270I)$\beta_1\gamma_{2L}$	13±8	1.1±0.1	5908±1719	94±4
α_2(A291W)$\beta_1\gamma_{2L}$	1.7±0.4	1.4±0.2	3683±765	102±4
$\alpha_2\beta_1$(S265I)γ_{2L}	67±8	1.4±0.1	6555±1755	96±1
α_2(S270I)β_1(S265I)γ_{2L}	8±2	1.0±0.1	4293±1605	104±3
$\alpha_2\beta_1\gamma_{2L}$(S280I)	52±6	1.2±0.04	14190±1916	98±1
$\alpha_2\beta_1\gamma_{2L}$(S301W)	53±13	1.3±0.1	13025±3460	98±0.4
$\alpha_2\beta_1\gamma_{2L}$(S280I/S301W)	25±4	1.6±0.1	10310±1366	101±1

Values are mean±s.e.mean, $n=3-6$ oocytes. GABA EC_{50} Hill slope and maximal response were calculated from nonlinear regression analysis as described in 'Methods'.

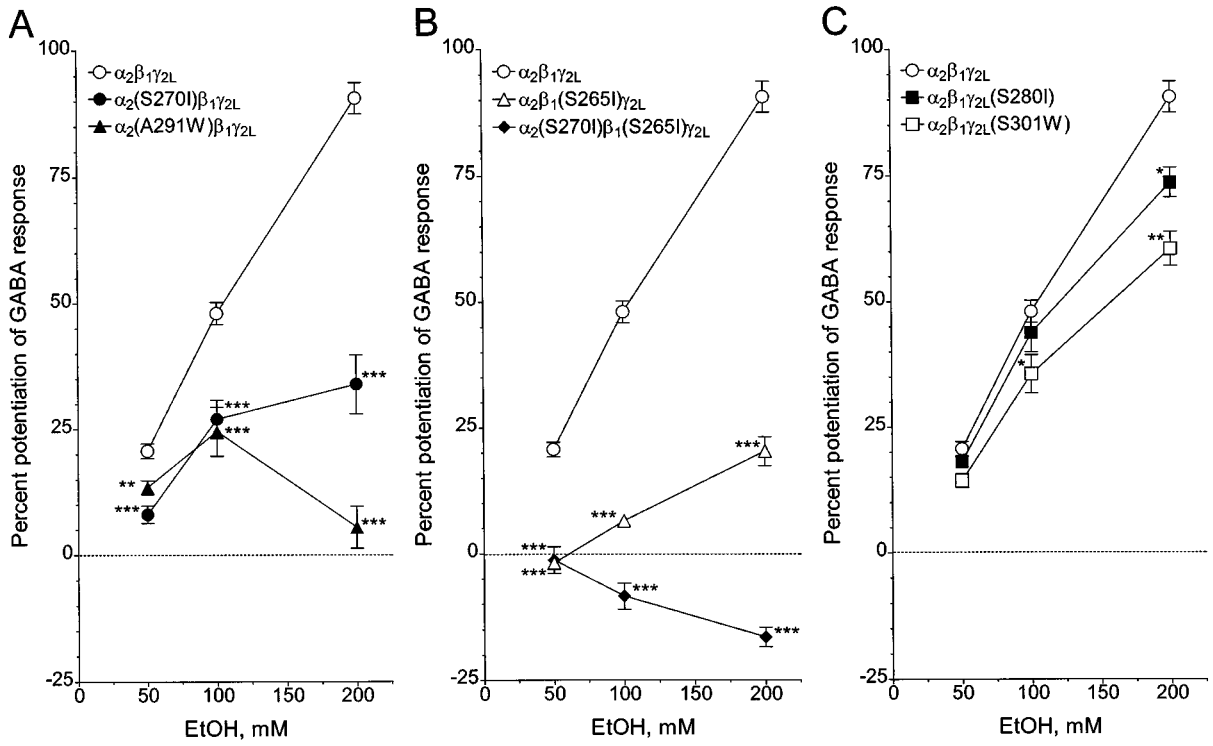

Figure 3 The effects of mutations in TM2 and TM3 in individual GABA$_A$ receptor subunits on the action of ethanol in *Xenopus* oocytes. (A) Oocytes expressing wild-type $\alpha_2\beta_1\gamma_{2L}$, mutant α_2(S270I)$\beta_1\gamma_{2L}$ or α_2(A291W)$\beta_1\gamma_{2L}$ receptors were preincubated with ethanol (50, 100 and 200 mM) for 2 min before being coapplied with EC_5 of GABA for 20–30 s. Same symbols as those in Figure 2 are used for presenting the values for each combination of receptor. (B) The values of potentiation are shown for mutant $\alpha_2\beta_1$(S265I)γ_{2L} and α_2(S270I)β_1(S265I)γ_{2L} receptor. The values for wild-type $\alpha_2\beta_1\gamma_{2L}$ are from (A). (C) The values of potentiation are shown for mutant $\alpha_2\beta_1\gamma_{2L}$(S280I) and $\alpha_2\beta_1\gamma_{2L}$(S301W) receptor. The values for wild-type $\alpha_2\beta_1\gamma_{2L}$ are from (A). *$P<0.05$, **$P<0.01$ and ***$P<0.001$, compared to wild-type $\alpha_2\beta_1\gamma_{2L}$ using ANOVA with Bonferroni's comparison *post hoc* test. All values are presented as mean±s.e.mean from 5–24 oocytes. In some cases, the error bars are smaller than the points.

Results

TM2 or TM3 mutations (S270I or A291W) in the α_2 subunit made the receptor 10 or 30 times more sensitive to GABA, respectively, as compared with wild-type receptors composed of human $\alpha_2\beta_1\gamma_{2L}$ subunits (Figure 2A). On the other hand, mutation of S265I in the β_1 subunit did not alter the sensitivity of the receptor to GABA (Figure 2B). Moreover, in the γ_{2L} subunit, neither the mutation of S280I nor S301W affected the apparent affinity for GABA (Figure 2B). A summary of the EC_{50} concentrations of GABA and the Hill slope for wild-type and mutant GABA$_A$ receptors is shown in Table 1.

Coexpression of mutant β_1(M286W) with wild-type α_2 and γ_{2L} subunits produced a receptor with unusual gating properties. An outward current was obtained from the application of picrotoxin to oocytes expressing mutant $\alpha_2\beta_1$(M286W)γ_{2L} receptors, i.e. in the direction opposite to that of the normal GABA-induced current (data not shown). Because picrotoxin closes GABA$_A$ receptor chloride channels, this result indicates that these mutant receptors formed tonically open channels. This subunit combination was not studied further.

We next compared the effects of ethanol on wild-type and mutant GABA$_A$ receptors. As shown in Figure 3A–C, ethanol produced up to 90% potentiation of the GABA-induced current for wild-type $\alpha_2\beta_1\gamma_{2L}$ GABA$_A$ receptors expressed in oocytes. Using an EC_5 concentration of GABA, we obtained 21 ± 1% potentiation by 50 mM ethanol, 48 ± 2% potentiation by 100 mM ethanol and 91 ± 3% potentiation by 200 mM ethanol. Coexpression of mutant α_2(S270I) or α_2(A291W) with wild-type β_1 and γ_{2L} subunits diminished the potentiation by all concentrations of ethanol (Figure 3A).

There was also a decrease in ethanol potentiation with mutant $\alpha_2\beta_1$(S265I)γ_{2L} receptors (Figure 3B), although this mutation did not affect the apparent affinity of the receptors for GABA (Figure 2B). Furthermore, receptors formed by this mutant β_1(S265I) subunit with the mutant α_2(S270I) and wild-type γ_{2L} subunits showed no potentiation by ethanol and even displayed significant inhibition ($P<0.01$, compared to the response produced by GABA EC_5 using paired, two-tailed t-test) by 200 mM ethanol (Figure 3B). This α_2(S270I)β_1(S265I)γ_{2L} receptor showed sensitivity to GABA that was similar to the mutant α_2(S270I)$\beta_1\gamma_{2L}$ receptor (Table 1). We did not examine the pharmacology of the mutant $\alpha_2\beta_1$(M286W)γ_{2L} receptor because of its unusual gating properties, and because oocytes in which these mutant receptors were expressed did not show stable currents or GABA responses.

Mutant $\alpha_2\beta_1\gamma_{2L}$(S280I) or $\alpha_2\beta_1\gamma_{2L}$(S301W) receptors closely resembled wild-type GABA$_A$ receptors (Figure 3C). Therefore, we next made a double mutation of S280I/S301W in the γ_{2L} subunit. For the GABA concentration-response curve, a small change was observed with the mutant $\alpha_2\beta_1\gamma_{2L}$(S280I/S301W) receptors (Figure 4, inset); nonlinear regression analysis yielded a slightly lower EC_{50} for GABA (Table 1). This double mutation also significantly reduced potentiation by 200 mM ethanol (Figure 4, bar graph); the decreased potentiation produced by this mutation was similar to that produced by a single mutation, S301W. Potentiation by 200 mM ethanol for the wild-type in this experiment, was somewhat lower (70 ± 3%) than that shown in Figure 3, apparently due to variability among batches of oocytes. We also examined the potentiation produced by 1 μM flunitrazepam, but there was no difference in flunitrazepam action between $\alpha_2\beta_1\gamma_{2L}$ and $\alpha_2\beta_1\gamma_{2L}$(S280I/S301W) (data not shown).

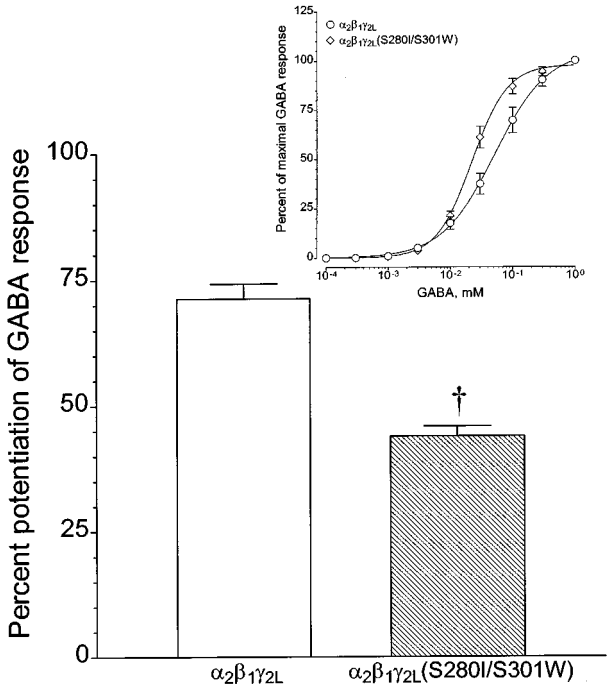

Figure 4 The effect of a double mutation in TM2/TM3 of γ_{2L} subunit on the potentiation by ethanol. Oocytes expressing wild-type $\alpha_2\beta_1\gamma_{2L}$ or mutant $\alpha_2\beta_1\gamma_{2L}$(S280I/S301W) receptors were incubated with 200 mM ethanol for 2 min followed by the coapplication with an EC_5 of GABA for 20–30 s. Values are presented as mean$\pm$s.e.mean from 17–38 oocytes. †$P<0.0001$, compared to wild-type $\alpha_2\beta_1\gamma_{2L}$ using unpaired, two-tailed t-test. Inset. GABA concentration-response curve for this mutant receptor is shown together with the one for wild-type, which is from Figure 2. Values are presented as mean$\pm$s.e.mean from five oocytes and nonlinear regression analysis of those curves was performed as described in 'Methods' and given in Table 1. In some cases, the error bars are smaller than the points.

Discussion

The aims of the present study were to investigate (i) the effect of mutations in the corresponding regions in TM2 and TM3 of α, β and γ subunits of the GABA$_A$ receptor on the potentiation by ethanol; (ii) the effect of mutations in individual GABA$_A$ receptor subunits on potentiation by lower as well as high concentrations of ethanol; (iii) the role of the γ subunit for the function of the GABA$_A$ receptor in combination with wild-type and/or mutant α and β subunits. Mutation of TM2 or TM3 sites in the γ_{2L} subunit produced a small reduction in the potentiation by ethanol, but had much less effect than did the analogous mutations in α_2 and β_1 subunits. The double mutation of S280I/S301W in the γ_{2L} subunit also reduced the potentiation by ethanol, but the degree of reduction was similar to that produced the single mutation of S301W at 200 mM ethanol. This double mutation did not affect the potentiation of receptor function by a benzodiazepine, flunitrazepam, the action of which requires the presence of the γ subunit (Pritchett *et al*., 1989). These results suggest that, for the potentiation by ethanol in the heteromeric GABA$_A$ receptor composed of the α_2, β_1 and γ_{2L} subunits, the amino acids in TM2 and/or TM3 of the α_2 and β_1 subunits are more important than are those of the γ_{2L} subunit.

Previous studies (Mihic *et al*., 1997; Ye *et al*., 1998) tested only 200 mM ethanol, a concentration not likely to be encountered *in vivo* (Deitrich & Harris, 1996), and we therefore asked whether those mutations also inhibited the action of lower (i.e. sub-anaesthetic) concentrations of ethanol. The

effect of mutations on the potentiation by 50 mM ethanol appeared smaller than that by 200 mM ethanol (Figure 3). Therefore, we calculated the ethanol effect on the mutant receptors as a percentage of the wild-type response, using wild-type data obtained from the same batch of oocytes. For all mutations tested, we found that the mutation had a similar effect at 50 or 200 mM ethanol (data not shown). Therefore, we conclude that all mutations affect the potentiation produced by not only a high concentration, but also lower concentrations of ethanol.

The present results suggest that the α_2 and β_1 subunits of the GABA$_A$ receptor are the primary sites for ethanol action, with the γ_{2L} subunit being less influential. The small effect of mutations on the γ_{2L} subunit, as compared to α_2 and β_1 subunits, in the present study may reflect the suggested stoichiometry of the receptor with two α and β subunits but only a single γ subunit (Chang *et al*., 1996; Tretter *et al*., 1997).

Mutations in TM2 or TM3 of the α_2 subunit altered the apparent affinity of GABA$_A$ receptors for GABA, but the mutation in TM2 of the β_1 subunit did not change the sensitivity to GABA. Mutations in neither TM2 nor TM3 in γ_{2L} subunit altered apparent affinity, but a small change in a GABA concentration-response curve was observed in TM2/TM3 double-mutation of γ_{2L} subunit. There is evidence for a GABA-binding site on the α subunit as mutations of Phe64 in the α_1 subunit produce marked decreases in the affinity of GABA$_A$ receptor agonists and antagonists for the receptor (Sigel *et al*., 1992), and this residue is photoaffinity-labelled by muscimol (Smith & Olsen, 1994). However, there is also evidence that the N-terminal regions of β subunit contain GABA-binding sites (Amin & Weiss, 1993), and it is possible that GABA binds at an interface between the α and β subunits. The changes in apparent affinity for GABA observed in the present study could be due to effects of the mutations on the binding affinity or on gating. Considering the distance of our mutations from the N-terminal regions that mediate GABA binding, the latter possibility seems most likely.

In summary, we suggest that ethanol binds in a cavity formed by TM2 and TM3 in the GABA$_A$ receptor subunits, and that the α_2 and β_1 subunits may be critical because they contain GABA-binding sites. The γ_{2L} subunit may also be less important than the α_2 or β_1 subunit, because only a single γ subunit may assemble in the pentameric oligomer. This suggestion of an ethanol binding cavity in GABA$_A$ receptors is supported by the recent study demonstrating that mutation of Ile307 and/or Trp328 (equivalent to the TM2 and TM3 residues) in the GABA ρ_1 subunit, to smaller amino acid residues (Ser and/or Ala, respectively) increased alcohol cutoff (Wick *et al*., 1998). Thus, our mutations to bigger residues in GABA$_A$ receptor α_2 and/or β_1 subunits may decrease the size of a cavity, resulting in the elimination of ethanol action. However, we should note that none of the experimental approaches used to date can provide direct, structural evidence for an ethanol binding cavity.

These studies extend previous work on homomeric glycine and GABA$_C$ receptors (Mihic *et al*., 1997; Wick *et al*., 1998) to heteromeric GABA$_A$ receptors coupled of three different subunits. For these three types of ligand-gated ion channels, similar regions of TM2 and TM3, near the extracellular face are critical for alcohol actions. Further work is required to determine if the site of alcohol action is similar in other ligand-gated ion channels. Finally, mutation of an amino acid in TM2 of the β_1 subunit does not change the action of GABA, but markedly affects the action of ethanol. Therefore, construction of transgenic mice or 'knock-in' mice with a mutant β subunit gene should allow us to determine which behavioural actions of ethanol require enhancement of GABA$_A$ receptor function *in vivo*.

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