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Tryptophan scanning mutagenesis in TM2 of the $GABA_A$ receptor α subunit: effects on channel gating and regulation by ethanol

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1 Each residue in the second transmembrane segment (TM2) of the human $GABA_A$ receptor α_2 subunit was individually mutated to tryptophan. The wild-type or mutant α_2 subunits were expressed with the wild-type human $GABA_A$ receptor β_2 subunit in *Xenopus* oocytes, and the effects of these mutations were investigated using two-electrode voltage-clamp recording.

2 Four mutations (V257W, T262W, T265W and S270W) produced receptors which were active in the absence of agonist, and this spontaneous open channel activity was blocked by both picrotoxin and bicuculline, except in the α_2(V257W)β_2 mutant receptor, which was not sensitive to picrotoxin.

3 Six mutations (V257W, V260W, T262W, T267W, S270W and A273W) enhanced the agonist sensitivity of the receptor, by 10–100 times compared with the wild-type $\alpha_2\beta_2$ receptor. Other mutations (T261W, V263W, L269W, I271W and S272W) had little or no effect on the apparent affinity of the receptor to GABA. Eight of the tryptophan mutations (R255, T256, F258, G259, L264, T265, M266 or T268) resulted in undetectable GABA-induced currents.

4 The S270W mutation eliminated potentiation of GABA by ethanol, whereas T261W markedly increased the action of ethanol. The T262W mutation produced direct activation (10% of maximal GABA response) by ethanol in the absence of GABA, while other mutations did not alter the action of ethanol significantly.

5 These results are consistent with a unique role for S270 in the action of ethanol within the TM2 region, and with models of $GABA_A$ receptor channel function, in which specific residues within TM2 are critical for the regulation of channel gating (S270, L264), while other residues (L269, I271 and S272) have little effect on these functions and may be non-critical structural residues.

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Abbreviations: GABA, γ-aminobutyric acid; TM, transmembrane (segment)

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain. The $GABA_A$ receptor is a receptor/chloride channel complex and is a member of the ligand-gated ion channel superfamily also including the glycine, $GABA_c$ (GABA ρ), 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) and nicotinic acetylcholine (nACh) receptors (Ortells & Lunt, 1995). Seven classes of $GABA_A$ receptor subunits (α_{1-6}, β_{1-4}, γ_{1-4}, δ, ε, π, θ) have been cloned to date (Barnard *et al*., 1998; Whiting *et al*., 1999), but most $GABA_A$ receptors in the brain appear to be composed of α, β and γ subunits with the consensus stoichiometry being $2\alpha{:}2\beta{:}1\gamma$ (Chang *et al*., 1996; Tretter *et al*., 1997; Farrar *et al*., 1999). The function of $GABA_A$ receptors can be markedly enhanced by pharmacologically-relevant concentrations of a number of classes of sedative, hypnotic and anaesthetic agents, including alcohols. Thus, the $GABA_A$ receptor is thought to be one of the major target proteins for the actions of alcohols in the brain (Samson & Harris, 1992; Franks & Lieb, 1994; Crews *et al*., 1996; Peoples *et al*., 1996; Diamond & Gordon, 1997; Valenzuela & Harris, 1997). The use of heterologous expression systems and receptor mutagenesis has allowed the investigation of ion channel function (Xu & Akabas, 1996), agonist binding (Amin & Weiss, 1993 and the molecular mechanisms of the action of ethanol on $GABA_A$ receptors (Mihic & Harris, 1996; Harris, 1999).

$GABA_A$ receptors expressed *in vitro* without the γ subunit remain sensitive to general anaesthetics and n-alcohols (Levitan *et al*., 1988; Pritchett *et al*., 1989; Harrison *et al*., 1993; Mihic *et al*., 1994a,b). This is an advantage for molecular and pharmacological studies on this receptor since the number of receptor cDNAs can be limited to two. Previous studies demonstrated that mutation of specific amino acids in the transmembrane segments (TM) 2 and 3 of the α or β subunit of the $GABA_A$ receptor can eliminate the action of ethanol without abolishing the response to GABA (Mihic *et al*., 1997; Ueno *et al*., 1999), and that the corresponding mutations introduced into the γ subunit have less effect (Ueno *et al*., 1999). These findings suggest that, in the $GABA_A$ receptor, S270 in TM2 of the α subunit may be critical for the actions of

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ethanol, but did not rule out a more general involvement of the entire TM2 segment.

In the present study, we replaced each TM2 residue of the $GABA_A$ receptor α subunit in turn with the large amino acid tryptophan, and used this 'tryptophan scanning mutagenesis' to probe the importance for ethanol sensitivity of each such residue. If the actions of ethanol are transduced by the entire TM2 segment, we reasoned that the introduction of the large volumes associated with the tryptophan side chain would cause large decrements in the activity of alcohol in a large number of these mutated receptors. If the action of ethanol were not widely distributed, but dependent upon specific binding interactions, the effects of tryptophan mutagenesis might be expected to be more discrete. We expressed each of the mutant α_2 subunits together with β_2 subunit in *Xenopus* oocytes and also modelled the TM2 region of the $GABA_2$ receptor α_2 subunit as an α-helix, in order to suggest potential structural effects of mutagenesis and to predict which residues might lie physically close to S270 (Figure 1B).

A

$GABA_A\,\alpha_1$	^{255}RTVFGVTTVLTMTTL**S**ISA^{273}
$GABA_A\,\alpha_2$	^{255}RTVFGVTTVLTMTTL**S**ISA^{273}
$GABA_A\,\beta_1$	^{250}RVALGITTVLTMTTI**S**THL^{268}
$GABA_A\,\beta_2$	^{250}RVALGITTVLTMTTI**N**THL^{268}
$GABA_A\,\gamma_2$	^{265}RTSLGITTVLTMTTL**S**TIA^{283}

B

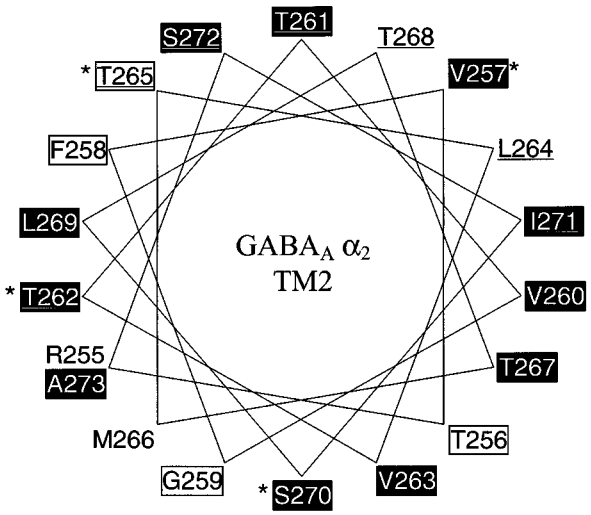

Figure 1 (A) Amino acid alignment of TM2 regions of human $GABA_A$ α_1, α_2, β_1, β_2 and γ_2 subunits. The bold letters show the position shown to be critical for alcohols and anaesthetics in $GABA_A$ receptors (Mihic *et al*., 1997). (B) Helical wheel diagram of TM2 of the $GABA_A$ receptor α_2 subunit. The view is looking down from the extracellular surface such that the right-handed α-helix of TM2 appears to be wound counterclockwise. The residues previously shown to be accessible from the pore of the channel are underlined (Xu & Akabas, 1996). Black boxes represent residues whose tryptophan-mutants showed GABA responses sufficient for study. Tryptophan mutations resulting in a weak GABA response (white boxes) or a failure of functional expression (no boxes) are indicated. The residues whose tryptophan-mutation produced receptors with spontaneous opening are indicated by asterisks. See 'Results' for details.

Methods

Adult female *Xenopus laevis* were obtained from *Xenopus* I (Ann Arbor, MI, U.S.A.); GABA and bicuculline methiodide from Research Biochemicals International (Natick, MA, U.S.A.); collagenase type 1A, picrotoxin, penicillin-streptomycin solution and gentamicin solution were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); ethanol was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY, U.S.A.). All other chemicals used were of reagent grade.

All tryptophan mutations were made in the cDNA encoding the human $GABA_A$ receptor α_2 subunit by use of the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) in the pCIS2 vector. The cDNA encoding the wild-type human $GABA_A$ receptor β_2 subunit was subcloned into the pCDM8 vector (Invitrogen, Carlsbad, U.S.A.). All point mutations were verified by double-stranded DNA sequencing.

Xenopus oocytes were isolated and injected with cDNAs (1.5–3.0 ng per 30 nl), and two-electrode voltage-clamp recordings were performed as described previously (Mihic *et al*., 1994b; 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 3 nM–1 mM GABA solutions to a single oocyte with an interval of 5 min at lower concentrations, or 15–25 min when desensitization was observed. We tested the ability of ethanol to enhance the effect of a concentration of GABA that produced 5% of a maximal effect (EC_5). There is a dependence of alcohol enhancement on the GABA concentration, with greater potentiation by ethanol being seen at the lower GABA concentrations (Mihic *et al*., 1994b). The GABA 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; this was immediately followed by a 20–30 s perfusion with GABA plus 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. All values are presented as mean ± standard error of the mean (s.e.mean) from 4–94 oocytes obtained from at least two different frogs. Curve fitting and estimation of EC_{50} values for concentration-response curves were performed using GraphPad Prism software (San Diego, CA, U.S.A.). Statistical analyses were also carried out by two-tailed, paired *t*-test using this software.

The amino acid sequences of transmembrane segments 2 and 3 (TM2 and TM3) were modelled as α-helices (Cutting *et al*., 1991) using the Biopolymer module of Insight II (MSI, San Diego, CA, U.S.A.). TM2 was positioned such that the polar residues that line the pore of the ion channel face to the left in Figure 7. TM3 was aligned anti-parallel with TM2 such that its axis was 11Å from TM2. The backbone atoms of the two alpha helices were tethered with a force constant of 10 kcal/Å^2 and side chain packing was optimized with Discover98 using the CFF91 potential energy functions. Replacing S270 with tryptophan and then re-optimizing the side chain packing while using a force constant of 10 kcal/Å^2 to tether the backbone atoms modelled the effect of the S270W mutation. The molecular volumes (van der Waals) of serine, tryptophan, and ethanol were calculated with Spartan (Wavefunction Inc. San Diego, CA, U.S.A.) using the AM1 semi-empirical parameters. The structures were optimized in an extended geometry.

Results

Nineteen different residues in the TM2 region of the $GABA_A$ receptor α_2 subunit were mutated to tryptophan. Four of these mutations, R255W ($n=27$), L264W ($n=28$), M266W ($n=25$) and T268W ($n=24$) did not produce any significant GABA-induced current (up to 10 mM GABA) when they were co-expressed with the wild-type β_2 subunit in *Xenopus* oocytes (Figure 1B). Three other mutations, T256W, F258W and G259W, resulted in receptors with weak responses to 1 mM GABA (Table 1). Because of the small or absent GABA response, these seven mutants were not investigated further in this study. The T265W mutant produced a weak response to 10 mM GABA (Table 1) and also made the receptor tonically open (see below).

Introduction of tryptophan into three positions made the receptor spontaneously open; coexpression of α_2(T262W), α_2(T265W) and α_2(T270W) with wild-type β_2 subunit produced receptors in which picrotoxin induced an outward current (Figure 2B–D and Table 1). Interestingly, bicuculline, (supposedly a competitive $GABA_A$ receptor antagonist), also produced an outward current on those mutant receptors. In receptors composed of α_2(T265W)β_2 or α_2(S270W)β_2, an inward current was observed upon removal of picrotoxin. On the other hand, V257W also produced tonic activity, which was blocked only by bicuculline, but not by picrotoxin (Figure 2A). In the α_2(V257W)β_2 receptor, GABA-induced current, as well as spontaneous current, was insensitive to picrotoxin (data not shown).

Compared to the wild-type $\alpha_2\beta_2$ receptors, the mutations of V257W, V260W, T262W, T267W, S270W and A273W made the resulting receptors 10 to 100 fold more sensitive to GABA (Figure 3 and Table 1). It should be noted that the α_2(T262W)β_2 receptor desensitized too rapidly to estimate the GABA EC_{50} and Hill coefficient from a complete concentration-response curve. Other mutations slightly altered (T261W, V263W or S272W) or did not affect (L269W or I271W) the apparent affinity of the receptor for GABA (Table 1).

Next we examined the effects of mutations on the actions of ethanol, using an EC_5 concentration of GABA. Across all the potentiation experiments for wild-type and mutant receptors reported, the actual percentage of maximal GABA response (EC values) for the test concentrations used were: wild-type $\alpha_2\beta_2$ (4.9±0.1%, total 100 experiments), α_2(T257W)β_2 (5.7±0.2%, seven experiments), α_2(V260W)β_2 (5.1±0.2%, 13 experiments), α_2(T261W)β_2 (5.2±0.3%, 12 experiments), α_2(T262W)β_2 (4.9±0.2%, 10 experiments), α_2(V263W)β_2 (5.0±0.1%, 12 experiments), α_2(T267W)β_2 (4.8±0.3%, 12 experiments), α_2(L269W)β_2 (4.9±0.3%, 10 experiments), α_2(S270W)β_2 (5.1±0.2%, 10 experiments), α_2(I271W)β_2 (5.5±0.3%, 10 experiments), α_2(S272W)β_2 (5.5±0.4%, eight experiments), α_2(A273W)β_2 (5.2±0.13%, eight experiments).

For the wild-type $\alpha_2\beta_2$ receptor, ethanol (200 mM) produced 94±3% potentiation without any direct activation (i.e., no effect of preincubation with ethanol alone) (Figures 4 and 5).

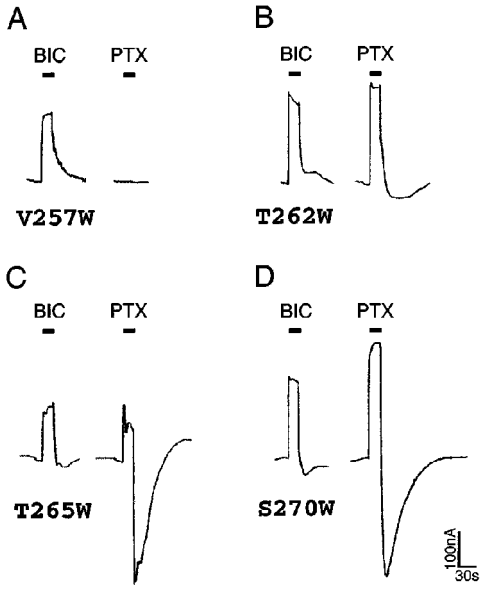

Figure 2 Sample tracings obtained from a single *Xenopus* oocyte expressing α_2(V257W)β_2 (A), α_2(T262W)β_2 (B), α_2(T265W)β_2 (C) or α_2(S270W)β_2 (D) demonstrate the effects of picrotoxin (PTX, 100 μM) and bicuculline (BIC, 50 μM) on the baseline current. An outward (upward) current indicates that these receptors are tonically open and an inward current was observed upon removal of picrotoxin from an oocyte expressing α_2(T265W)β_2 or α_2(S270W)β_2.

Table 1 Summary of spontaneous activities, GABA EC_{50}s, Hill coefficients and maximal responses produced by 1 mM GABA for wild-type and mutant $GABA_A$ receptors expressed in *Xenopus* oocytes

Mutation	*Spontaneous activity (maximal outward current, nA)*	*GABA EC_{50}, μM (95% confidence interval)*	*Hill coefficient (95% confidence interval)*	*Maximal response, nA*
T256W	–	ND	ND	78±15
V257W	+ (171±11)*	0.74 (0.63–0.88)	1.3 (1.0–1.5)	2076±303
F258W	–	ND	ND	29±5
G259W	–	ND	ND	59±12
V260W	–	2.4 (1.6–3.4)	0.7 (0.6–0.9)	2284±252
T261W	–	10 (8.1–13)	1.0 (0.8–1.2)	2966±443
T262W	+ (143±46)#	0.55 (0.46–0.65)	0.9 (0.7–1.0)†	2313±251
V263W	–	54 (42–67)	0.9 (0.8–1.0)	1137±90
T265W	+ (50±11)#	ND	ND	17±3‡
T267W	–	3.1 (2.5–4.0)	0.9 (0.7–1.0)	2751±382
L269W	–	21 (18–24)	1.2 (1.0–1.3)	2231±359
S270W	+ (161±13)#	0.20 (0.16–0.25)	1.0 (0.9–1.2)	1467±185
I271W	–	23 (19–29)	1.1 (0.9–1.3)	3571±684
S272W	–	9.0 (7.0–11)	1.2 (1.0–1.4)	1918±246
A273W	–	1.9 (1.6–2.3)	1.2 (1.0–1.4)	3167±665
Wild-type	–	31 (23–43)	0.9 (0.7–1.1)	5119±261

The EC_{50} and Hill coefficient values were obtained from 4–10 oocytes. The values of spontaneous activity and maximal response are presented as mean±s.e.mean obtained from all oocytes used in this investigation. ND, not determined; *response to 50 uM bicuculline; #response to 100 uM picrotoxin; †estimated from partial GABA concentration-response curves; ‡response to 10 mM GABA.

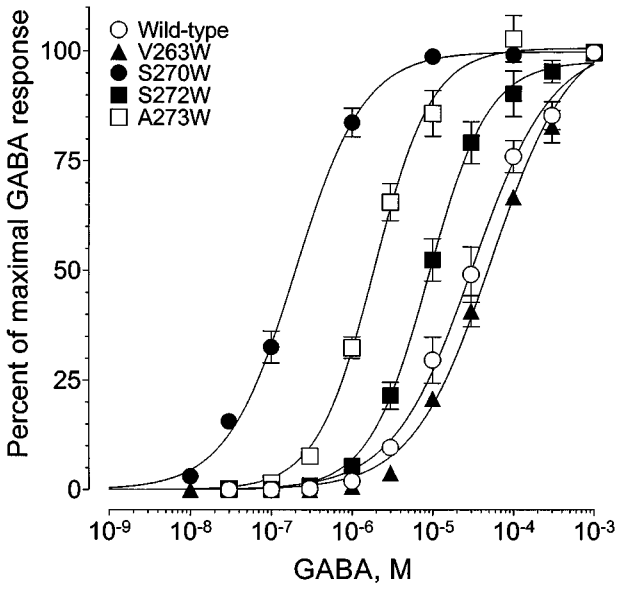

Figure 3 GABA concentration-response curves obtained from wild-type $\alpha_2\beta_2$ and α_2(V263W)β_2, α_2(S270W)β_2, α_2(S272W)β_2 and α_2(A273W)β_2 $GABA_A$ receptors expressed in *Xenopus* oocytes. Nonlinear regression analysis of the curves was performed as described in 'Methods', and the values of GABA EC_{50} and Hill coefficient are summarized in Table 1. Values are presented as mean ± s.e.mean from 4–10 oocytes. In some cases, the error bars are smaller than the points.

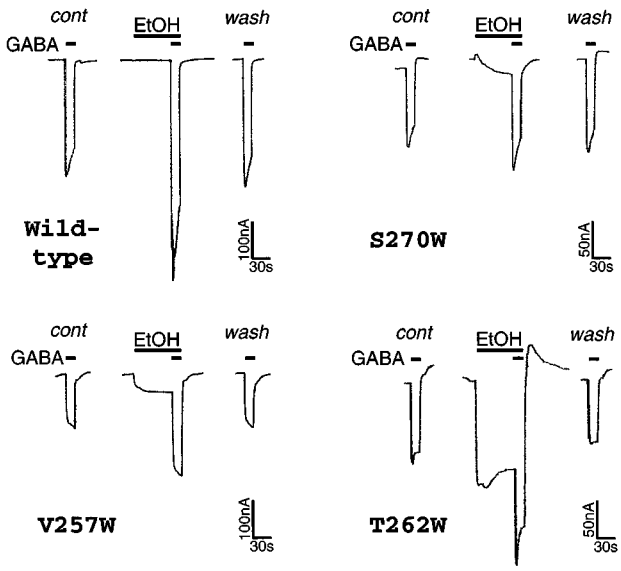

Figure 4 Sample tracings obtained from *Xenopus* oocytes expressing either the wild-type $\alpha_2\beta_2$ or mutant α_2(S270W)β_2, α_2(V257W)β_2, or α_2(T262W)β_2 $GABA_A$ receptors demonstrating the effects of ethanol (EtOH, 200 mM) on currents induced by GABA (EC_5). Ethanol was preapplied for 2 min before co-application of GABA, followed by a 15-min washout period.

The S270W mutation which has been reported to eliminate the potentiating action of isoflurane (Koltchine *et al*., 1999), also abolished (6 ± 5%) the ethanol action. A small degree of direct activation by ethanol (1.9 ± 0.2% of maximal GABA response) was obtained in the α_2(V275W)β_2 receptor, in which there was a slight reduction (to 64 ± 6%) in ethanol potentiation (Figure 5). The α_2(V263W)β_2 or α_2(T267W)β_2 receptors showed 48 ± 5% or 68 ± 5% potentiation of GABA-induced current, respectively. Interestingly, ethanol directly activated the mutant α_2(T262W)β_2 receptors to the extent of 10 ± 1% of

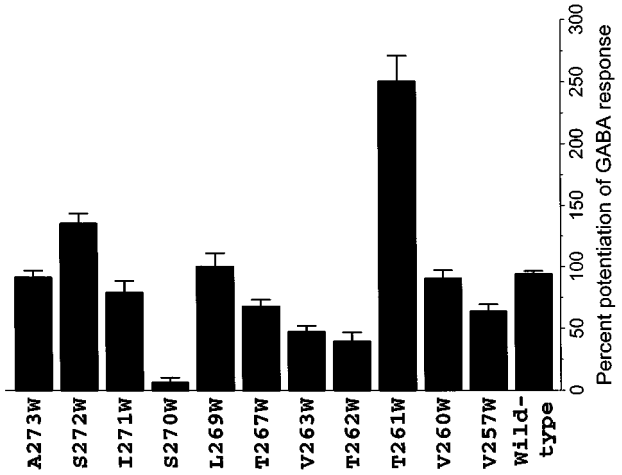

Figure 5 The effect of tryptophan-mutations in the $GABA_A$ receptor α_2 subunit on the potentiation by ethanol of the resultant receptors expressed in *Xenopus* oocytes. Oocytes co-expressing wild-type or tryptophan-mutant α_2 subunits with wild-type β_2 subunits were incubated with 200 mM ethanol for 2 min followed by coapplication with an EC_5 of GABA for 20–30 s. The values of potentiation by ethanol of the GABA-induced current are shown for the wild-type and mutant receptors. Each data represents the mean ± s.e.mean from 6–94 oocytes.

maximal GABA response, but the potentiation of GABA-induced current was reduced (to 40 ± 7%) in comparison to the wild-type receptor.

One mutant, α_2(T261W)β_2, demonstrated a very large potentiation (251 ± 21%) by 200 mM ethanol, without showing any direct effect of ethanol (Figures 5 and 6A). For this mutant we studied the potentiation by ethanol over a wide range of ethanol concentrations, and the T261W mutant showed significant potentiation at the concentration as low as 10 mM, compared to the wild-type that showed significant potentiation only at 50 mM and higher (Figure 6B). Other tryptophan-mutant receptors, such as α_2(V260W)β_2, α_2(L269W)β_2, α_2(I271W)β_2, α_2(S272W)β_2, or α_2(A273W)β_2, exhibited actions of ethanol that did not differ from the wild-type receptor.

Discussion

Both the Ser270 in the $GABA_A$ receptor α subunit and the corresponding residue (Ser267) in the glycine receptor α subunit have been previously reported to be critical for the actions of alcohols and volatile anaesthetics (Mihic *et al*., 1997; Krasowski *et al*., 1998). At this position the volume of the amino acid residue is negatively correlated with the potentiation produced by alcohols/anaesthetics; that is, substitution with larger amino acids at that site leads to decrease enhancement (Ye *et al*., 1998; Koltchine *et al*., 1999). The importance of side chain volume at this site is further underscored by the observation that substitution of small residues in TM2/TM3 regions of the GABA ρ_1 receptor increase the alcohol 'cutoff' toward longer chain alcohols, suggesting that alcohols may bind in a cavity formed in part by TM2 (Wick *et al*., 1998).

The S270W mutation, alone among the 19 mutations studied, completely eliminated the potentiation of GABA currents by ethanol. The V257W, T262W, V263W and T267W mutations reduced but did not abolish ethanol potentiation. We therefore conclude that S270 is the only amino acid residue that is absolutely critical for the action of ethanol in the TM2

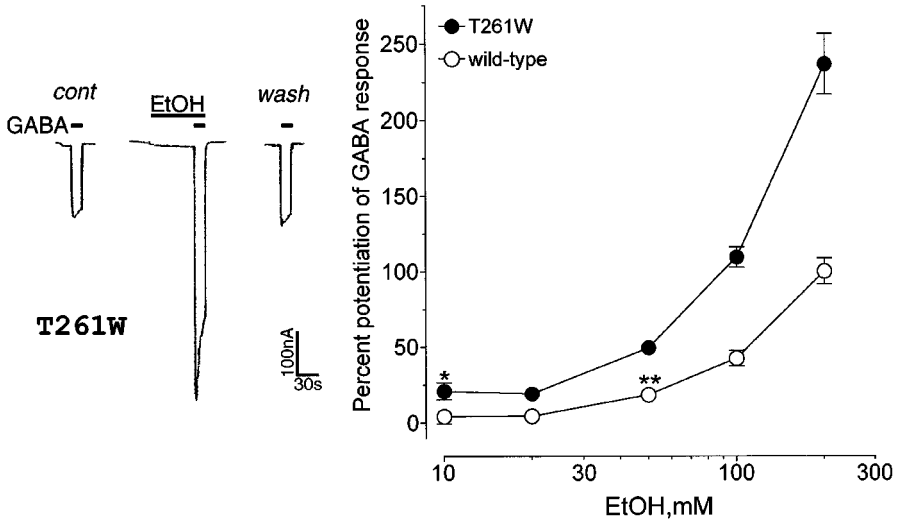

Figure 6 (A) Sample tracings obtained from an oocyte expressing α_2(T261W)β_2 demonstrate the effects of ethanol (200 mM) on currents induced by GABA (EC_5). Ethanol was preapplied for 2 min before co-application with GABA, followed by a 15-min washout period. (B) Potentiation by different concentrations of ethanol on currents induced by GABA (EC_5). All values are represented as mean ± s.e.mean from six oocytes. In some cases, the error bars are smaller than the points. For the mutant, the potentiation is significant (*$P<0.05$ using two-tailed, paired *t*-test) at concentrations of 10 mM and higher. For the wild-type receptor, the potentiation is significant (**$P<0.01$ using two-tailed, paired *t*-test) at concentrations of 50 mM and higher.

region of the $GABA_A$ receptor α_2 subunit. We also suggest that the slight reduction in ethanol potentiation of GABA response produced by the mutations of V263W or T267W may be because the positions of these residues are physically close to S270, as predicted from a helical wheel diagram of the TM2 region (Figure 1B). A similar reduction in the action of ethanol has been reported by the mutation of T264 in the glycine receptor α_1 subunit, which corresponds to the position of T267 in the $GABA_A$ receptor α_2 subunit (Mihic *et al*., 1997).

According to our helical wheel diagram, the position of V257 or T262 is not supposed to be close to S270, but tryptophan mutations at these positions resulted in a small decrease in ethanol action. This could be because spontaneous activities in the resultant receptors have a role in the reduced potentiation by ethanol. Recently Thompson *et al*. (1999) have reported that the mutation in TM2 of the $GABA_A$ receptor β subunit makes the resultant receptors tonically open, more sensitive to GABA and fail to potentiate the GABA response by positive allosteric modulators. They conclude that the positive allosteric modulation can be reduced by mutations that shift the receptor EC_{50} closer to the intrinsic binding affinity because no further leftward shift in an agonist dose-response relationship is possible. If our changes in ethanol actions were due only to changes in GABA sensitivity, then we would predict that there would be a relationship between GABA EC_{50} and the potentiation by ethanol among the mutants. However, we found no significant correlation between these two parameters for the mutants tested ($r^2=0.16$). Thus, our results suggest that changes in ethanol action produced by tryptophan mutations are mainly due to effects of the mutations on alcohol action rather than GABA action, although it remains possible that changes in GABA sensitivity and spontaneous activity in the mutant receptors also contribute to the differences in alcohol sensitivity.

Many of the mutants tested made the receptor more sensitive to GABA. Moreover, V257W, T262W, T265W or S270W mutants made the receptors spontaneously active and tryptophan mutations of an Ala residue in the TM3 region of $GABA_A$ receptor α_2 and β_1, or glycine receptor α_1 subunits also produce receptors with tonically open channels (Mihic *et al*., 1997; Ueno *et al*., 1999). Moreover, Lasalde *et al*. (1996) have reported that tryptophan substitutions into the TM4 region of the *Torpedo* nicotinic acetylcholine receptor stabilize the channel in the open state.

Taken together, these results suggest that the introduction of bulky residues within a transmembrane domain of ligand-gated ion channels might reduce the free energy difference between the more stable closed and less stable open states. This reduction in the free energy difference could also interpret the direct activation by ethanol, which was observed remarkably in the T262W-containing receptor (Figure 4). Direct effects of ethanol in the absence of GABA were found only with receptors that showed spontaneous activities (T265W was too difficult to express and ethanol actions were not determiend).

Picrotoxin and bicuculline appear to inhibit the spontaneous opening of all mutants, except for the V257W mutation, which made the receptor insensitive to picrotoxin. It has been reported that the corresponding position in the $GABA_A$ receptor α_1 subunit (also V257) may be a binding site of picrotoxin (Xu *et al*., 1995), and our data would suggest that the tryptophan mutation interferes with the binding of picrotoxin at this position. A possible interpretation for the closing of the channel by bicuculline in several of the mutant receptors studied here is that bicuculline acts as an allosteric inhibitor, rather than a true competitive antagonist, so that it can stabilize the channel in the closed state, as suggested by Chang & Weiss (1999) using other spontaneously opening $GABA_A$ receptors.

The rebound currents following removal of picrotoxin were an unusual feature of some of the mutant receptors. These currents were especially noticeable in the α(T265W) and α(S270W) mutants (Figure 2C,D). A plausible explanation for this behaviour may be provided by a consideration of a simple three-state model for these receptors:

$$C \longleftrightarrow O \longleftrightarrow D$$

Under 'baseline' conditions, in the absence of agonist, the wild-type receptors are all in the closed (C) state, which is energetically highly stable relative to the open (O) and desensitized (D) states. Hence picrotoxin produces no current

in wild-type receptors. Binding of agonist to the C state then promotes transition to the stabilized O and D states and allows current to flow, until agonist is removed, prompting a return to the C state. We hypothesize that the energetics of the mutant receptors are such that, even in the absence of agonist, they exist in an equilibrium between closed (C), open (O) and desensitized (D) states, so that there is significant 'spontaneous' inward current through receptors in the O state. The addition of picrotoxin results in what appears to be an outward current (Figure 2D), in reality reflecting a rapid block by the drug of the standing inward current. It is presumed that picrotoxin promotes a transition from the O and D states to the C state. The subsequent removal and very rapid unbinding of picrotoxin then allows the receptors to undergo relaxation from the C state to the O state, resulting in the large inward 'rebound' currents we observed (Figure 2C,D). The subsequent 'fade' of the rebound currents reflects the slower kinetics of relaxation from the O state to the D state (Figure 2D), until the original equilibrium between the O and D states is re-established.

Rebound currents are not observed with bicuculline. It is likely that the kinetics of unbinding of bicuculline are slower (as seen in, for example, the experiment shown in Figure 2A, from the V257W mutant) relative to the C-O and O-D transitions, so that these cannot be observed independently following removal of the drug. Unbinding of bicuculline is therefore rate-limiting and the return to equilibrium state appears to follow a simpler monophasic trajectory.

It is particularly interesting that the T261W mutation produces a striking increase in the potentiation by ethanol, resulting in significant effects of 10 mM ethanol, the lowest concentration tested. There is evidence that T261 faces into the channel (Xu & Akabas, 1996), and is positioned horizontally opposite to S270 according to the helical wheel diagram (Figure 1B). One possible interpretation of our results would be that the T261W mutation changes the conformation of the protein in the region bounded by S270 and thereby increases binding of ethanol, perhaps stabilizing the channel open state in the presence of the drug.

In an attempt to visualize the effect of tryptophan mutation at position S270, TM2 and TM3 domains of the $GABA_A$ receptor wild-type α_2 and α_2(S270W) subunits were modelled as anti-parallel α-helices (Figure 7). The positions of TM2 and TM3 were modelled based on exposure to hydrophobic photoactivatable probes in the lipid region of the acetylcholine receptor: TM1 was only slightly labelled, TM2 was not labelled, TM3 was partially labelled, and TM4 was most labelled (Blanton & Cohen, 1994). In contrast, exposure of cysteine mutants of TM1 and TM2 of the acetylcholine receptor to a water-soluble probe revealed that TM2 was labelled in a manner consistent with an α-helix whereas TM1 was labelled in an irregular manner (Akabas & Karlin, 1995). In addition, activation by GABA increases the accessibility of residues on TM3 of $GABA_A$ receptors to water-soluble probes (Williams & Akabas, 1999).

The model of the TM2-TM3 region in wild-type α_2 subunit shows that the region between S270 (TM2) and TM3 contains a small cavity that may not be filled by side chains of adjoining helices. In contrast, the model of the S270W mutation shown on the right hand side of the figure demonstrates that the side chain of tryptophan completely occupies this cavity, which could eliminate occupation of the putative capacity by ethanol. Our calculations of the molecular volumes (van der Waals) of serine and tryptophan showed that tryptophan (224 $Å^3$) is 114 $Å^3$ larger than serine (110 $Å^3$). If ethanol (molecular volume of 70 $Å^3$) cannot be accommodated within the remaining resultant cavity, the volume of the putative cavity in the wild-type receptor can be estimated as 180 $Å^3$, similar to estimates ($\sim$200 $Å^3$) suggested by recent work with the volatile anaesthetic ether, isoflurane (Koltchine *et al*., 1999).

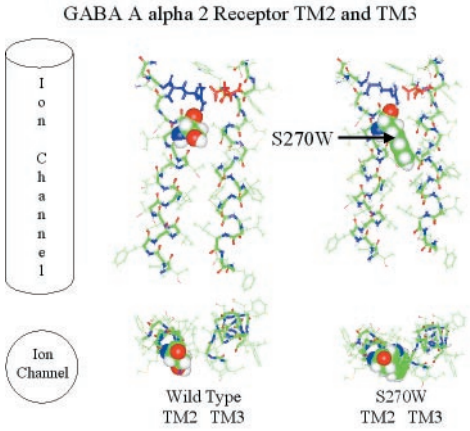

Figure 7 Transmembrane segments 2 and 3 (TM2 and TM3) of $GABA_A$ receptor α_2 subunit were modelled as α-helices as described in the text. The outline of an ion channel at the far left side of the figure suggests the consensus relationship of TM2 to the pore of the ion channel. A view in the plane of the membrane of the TM2 and TM3 segments of the wild-type receptor is shown (top left panel) as a space-filling surface (van der Waals volume) to emphasize the small cavity between S270 and TM3. In contrast, the top right panel shows the effect of the S270W mutation and only W270 is shown as a space-filling surface to demonstrate that the side chain of tryptophan could fill the cavity present in the wild-type. Views from the extracellular side of the TM2 and TM3 α-helices of the wild-type (bottom left panel) and the S270W mutation (bottom right panel) are shown.

In summary, the introduction of individual tryptophan mutations into the TM2 region changes the gating properties of $GABA_A$ receptors significantly in many of the mutant receptors, while the S270W mutant was unique in eliminating the action of ethanol. Our simple calculations provide surprising agreement between experimental data and theoretical prediction that alcohols may potentiate GABA responses by binding in the region of S270. However, it is important to note the alternative explanation that ethanol binds elsewhere on the receptor and that the TM2 region alters gating of the receptor thereby changing actions of ethanol. Further investigation is required to determine if alcohols bind to these amino acids in the TM2 and TM3 regions.

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