

## Mutations of $\gamma$ -aminobutyric acid and glycine receptors change alcohol cutoff: Evidence for an alcohol receptor?

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**ABSTRACT** Alcohols in the homologous series of  $n$ -alcohols increase in central nervous system depressant potency with increasing chain length until a “cutoff” is reached, after which further increases in molecular size no longer increase alcohol potency. A similar phenomenon has been observed in the regulation of ligand-gated ion channels by alcohols. Different ligand-gated ion channels exhibit radically different cutoff points, suggesting the existence of discrete alcohol binding pockets of variable size on these membrane proteins. The identification of amino acid residues that determine the alcohol cutoff may, therefore, provide information about the location of alcohol binding sites. Alcohol regulation of the glycine receptor is critically dependent on specific amino acid residues in transmembrane domains 2 and 3 of the  $\alpha$  subunit. We now demonstrate that these residues in the glycine  $\alpha 1$  and the  $\gamma$ -aminobutyric acid  $\rho 1$  receptors also control alcohol cutoff. By mutation of Ser-267 to Gln, it was possible to decrease the cutoff in the glycine  $\alpha 1$  receptor, whereas mutation of Ile-307 and/or Trp-328 in the  $\gamma$ -aminobutyric acid  $\rho 1$  receptor to smaller residues increased the cutoff. These results support the existence of alcohol binding pockets in these membrane proteins and suggest that the amino acid residues present at these positions can control the size of the alcohol binding cavity.

Although ethanol and the longer-chain alcohols have no known specific binding sites within the central nervous system, attempts to correlate the pharmacological effects of alcohols with their effects on lipid structure have been mostly unsatisfactory (1–3). Recently, studies on the mechanisms of action of alcohols have shifted in emphasis to a relatively small number of central nervous system targets; chief among them are the ligand-gated ion channels (LGICs). Neurotransmitter receptors for the inhibitory amino acids  $\gamma$ -aminobutyric acid (GABA) and glycine are LGICs that have been intensively studied as likely targets of ethanol action in the brain and spinal cord (3–8).

Direct measurement of binding of alcohol to these receptors by physical methods is not yet possible, but an indication that such binding sites exist is provided by the phenomenon of alcohol “cutoff.” It is well known that the potencies of  $n$ -alcohols in producing central nervous system depression increase with increasing carbon chain length, but only up to a certain size (the cutoff), after which alcohols with longer carbon chains decline in potency and efficacy (9–11) or are equally potent with the  $(n - 1)$ -alcohol. The proposed mechanism for the cutoff phenomenon is that there exists a critical

alcohol binding site of finite size that will accommodate only alcohols below a certain limit of molecular volume. The various LGICs that are regulated by alcohols also demonstrate cutoff behavior. Of crucial interest is the observation that the cutoff varies among this group of membrane proteins (as expressed in *Xenopus* oocytes), so that the cutoff (in the  $n$ -alkanol series  $C_nH_{2n+1}OH$ ) for type A GABA (12) and glycine (13) receptors is at  $n = 10$  to 12 and the cutoff for *N*-methyl-D-aspartate, kainate, and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-gated receptors is at  $n = 7$  or 8 (ref. 12). Because the ability of any particular alcohol to produce an effect may depend on the water solubility (3, 8), we have chosen to define cutoff as the point at which the potency of the  $n$ -alcohol no longer exhibits an increase with increasing carbon-chain length. The smallest cutoff yet reported for a LGIC is seen in the ATP-gated ion channels, where the cutoff falls at  $n = 3$  (ref. 14). These data suggest that the size of the alcohol binding pockets within the family of LGIC proteins varies substantially and that it is influenced by the details of protein structure.

The basis of our experimental approach is that the homomeric  $\alpha 1$  glycine receptor (Gly-R- $\alpha 1$ ) exhibits an alcohol cutoff at 10 (ref. 13), whereas the phylogenetically related GABA  $\rho 1$  receptor (GABA-R- $\rho 1$ ) shows a cutoff at  $n = 7$  (ref. 15). Recent work suggests that residues within the second and third transmembrane domains (TMs), TM2 and TM3, of these receptors are critical for regulation of function by alcohols (16, 17) and that alcohol action can be altogether eliminated by select mutations at either of two of these positions. It is likely that these amino acids do not line the ion-channel pore but may form a hydrophobic pocket near the extracellular surface of the receptor (16). Interestingly, the action of the intravenous anesthetic etomidate at type A GABA receptors has recently been shown to be highly dependent upon a single amino acid residue within TM2 of the  $\beta$  subunit, which is in a homologous position to the TM2 residue implicated in alcohol regulation of these receptors (18). If the alcohols do exert their action on GABA and glycine receptors by binding in this hypothetical pocket formed by TM2 and TM3, then the  $n$ -alcohol cutoff data suggests that this pocket is smaller in size in the GABA-R- $\rho 1$  than in the Gly-R- $\alpha 1$ . Thus, a crucial test of this binding site hypothesis would be to determine whether the alcohol cutoff in these receptors can be altered by exchanging fragments between the two or by mutating key individual amino acid residues. In this light, it is important to note that the goal in exchanging key fragments or in mutating key amino acid

Abbreviations: ADH, alcohol dehydrogenase; GABA,  $\gamma$ -aminobutyric acid; GABA-R- $\rho 1$ , GABA  $\rho 1$  receptor subunit; Gly-R- $\alpha 1$ , glycine  $\alpha 1$  receptor subunit; LGIC, ligand-gated ion channel; TM, transmembrane domain.

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residues is not to eliminate the effects of alcohols, as has already been demonstrated for ethanol (16), but to increase or decrease the chain length ( $n$ ) of the  $n$ -alcohol that fails to have a more potent effect on the particular LGIC than the ( $n - 1$ )-alcohol. We examined the actions of the alcohols on the Gly-R- $\alpha 1$  and GABA-R- $\rho 1$ , as well as on chimeras and mutants of these LGICs, by electrophysiological experiments conducted with LGICs expressed in *Xenopus* oocytes. Although both the Gly-R- $\alpha 1$  and GABA-R- $\rho 1$  were examined, the GABA-R- $\rho 1$  was studied in greater detail.

## MATERIALS AND METHODS

Human cDNAs were subcloned into the pCIS2 or pBK-CMV (Stratagene) vectors. The pBK-CMV vector was modified by removal of the *lac* promoter and the *lacZ* ATG. Chimeras C1 and C3 were constructed as described (16). Isolation of oocytes, cDNA injections (1.5 ng in 30 nl), and two-electrode voltage clamp recordings from *Xenopus* oocytes (at a holding potential of  $-70$  mV) were performed as described (15, 19). Glycine (30-sec application) or GABA (3-min application) were dissolved in modified Barth's saline (MBS) and washed out for 5 min (glycine) or 12 min (GABA). Ethanol ( $n = 2$ ) was purchased from Aaper Alcohol and Chemical (Shelbyville, KY).  $n$ -Alcohols ( $C_nH_{2n+1}OH$ ) with  $n > 2$  were purchased from Sigma. The  $n$ -alcohols ethanol up to hexanol were dissolved directly in MBS.  $n$ -Alcohols with  $n > 6$  were first dissolved in dimethyl sulfoxide (DMSO), then diluted in MBS to a final DMSO concentration not exceeding 0.05%; the  $n$ -alcohol/MBS solutions were sonicated for 30 min to facilitate the equilibration with MBS. Oocytes were perfused with the alcohols for 2 min before coapplication of the agonist, to allow for complete equilibration of the oocytes with the alcohol. A 15-min washout period was allowed after application of the alcohol/agonist solutions. All alcohol concentrations given are corrected for loss occurring during bath perfusion of the oocytes (12). The agonists glycine and GABA were applied at concentrations producing 5–10% of the maximal effect; when identifying the concentration of agonist producing this effect, 1 mM GABA or 1 mM glycine was used to produce a maximal current. Each data point was obtained with 4–10 oocytes obtained from at least two frogs.

Site-directed mutagenesis of the GABA  $\rho 1$  or Gly  $\alpha 1$  cDNAs in the pCIS2 or modified pBK-CMV vector was performed by using either the QuikChange site-directed mutagenesis kit (Stratagene) or the USE kit (Pharmacia Biotech). All point mutations were confirmed by double-stranded DNA sequencing.

Computer modeling of the wild-type and mutated GABA-R- $\rho 1$  was accomplished with DISCOVER 97 (MSI) using the CFF91 force field. Molecular volumes of amino acid residues and the  $n$ -alcohols were calculated with the program SPARTAN (Wavefunction, San Diego, CA) using the AM1 semiempirical parameters.

## RESULTS

The potentiation of Gly-R- $\alpha 1$  activity and the inhibition of GABA-R- $\rho 1$  activity by various concentrations of the  $n$ -alcohols ( $C_nH_{2n+1}OH$ ) were studied in *Xenopus* oocytes (Fig. 1A). For all alcohols, we tested the highest concentrations that could be applied without exceeding the water solubility of the alcohols or without producing toxic effects such as changes in membrane conductance unrelated to GABA or glycine receptors (20, 21). Because of very limited water solubility, alcohols longer than  $n = 12$  could not be tested. We found that the alcohol cutoff (defined as the chain length at which the potency of the  $n$ -alcohols no longer increases) for the Gly-R- $\alpha 1$  is at 10. As shown in Fig. 1A Upper, 0.05 mM hexanol potentiates the effect of glycine on the Gly-R- $\alpha 1$  by about 25%. At the same

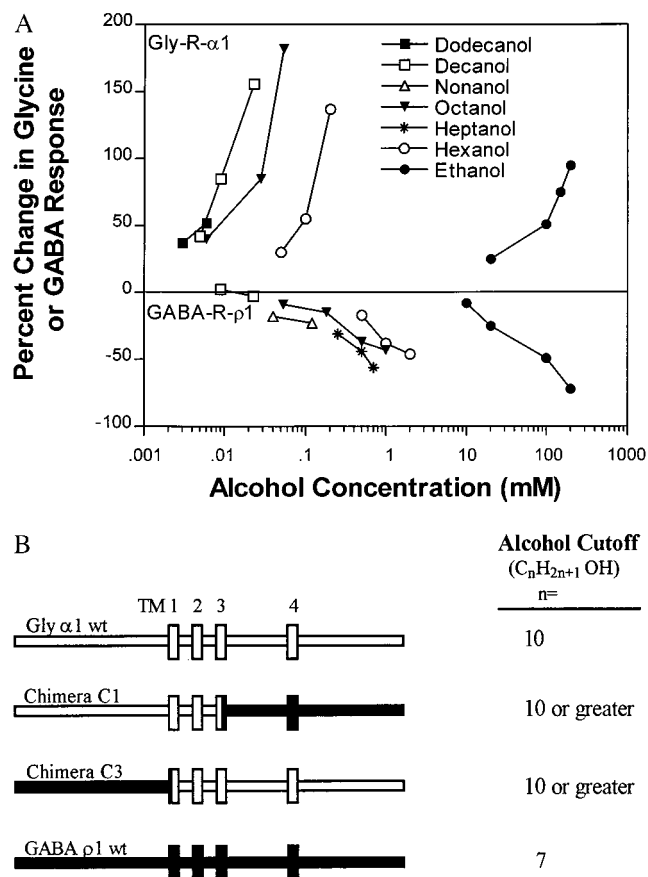


Fig. 1. (A) Determination of alcohol cutoff in homomeric Gly-R- $\alpha 1$  (Upper) and GABA-R- $\rho 1$  (Lower) expressed in *Xenopus* oocytes. The  $n$ -alcohols potentiate Gly-R- $\alpha 1$  and inhibit GABA-R- $\rho 1$  function. The alcohol cutoffs differ between the two receptors: Gly-R- $\alpha 1$  has a cutoff of decanol and GABA-R- $\rho 1$  has a cutoff of heptanol. (Upper) Data are adapted from Mascia *et al.* (13) and were gathered from 5 to 8 oocytes. (Lower) Data are adapted from Mihic and Harris (15) and were gathered from 3 to 11 oocytes, except for data on the effects of decanol on the GABA-R- $\rho 1$ , which were determined from 4 oocytes in the present study. Error bars are omitted for clarity; in each case, the SEM for each point was typically less than 10% of the mean. (B)  $n$ -Alcohol cutoffs of wild-type Gly-R- $\alpha 1$ , GABA-R- $\rho 1$ , and C1 and C3 chimeric receptors. Schematic representations of the receptors are shown on the left; the four transmembrane domains are depicted as vertically oriented rectangles. Gly-R- $\alpha 1$  sequences are open; GABA-R- $\rho 1$  sequences are solid. Interfaces between open and solid bars indicate chimera junction sites. For chimera C1, the junction site is immediately after Gly-R- $\alpha 1$  E300; for chimera C3, the junction site is after GABA-R- $\rho 1$  Val-274 (numbering is based on the amino acid sequence after signal peptide cleavage). The  $n$ -alcohol cutoffs of the wild-type receptors (Gly-R- $\alpha 1$  and GABA-R- $\rho 1$ ) were determined from A. The  $n$ -alcohol cutoff of chimera C1 was estimated to be 10 or greater because of the similar increase in potency in the  $n$ -alcohol series ethanol, hexanol, and decanol for both chimera C1 and the Gly-R- $\alpha 1$ . The  $n$ -alcohol cutoff of chimera C3 was based on analysis of the effects of ethanol, hexanol, octanol, decanol, and dodecanol, all of which exhibited increased potency over the previous alcohol. All of the receptors were expressed in *Xenopus* oocytes and tested with an EC<sub>5-10</sub> concentration of GABA or glycine.

concentration, octanol is much more effective than hexanol and potentiates the glycine-gated response by about 150%; thus octanol is much more potent than hexanol on the Gly-R- $\alpha 1$  (Fig. 1A). Likewise, decanol is more potent on the Gly-R- $\alpha 1$  than is octanol, because the concentration of decanol required to produce more than 150% potentiation of the glycine-gated response of the Gly-R- $\alpha 1$  is just over 0.02 mM. However, similar concentrations of decanol and dodecanol produced a potentiation of the Gly-R- $\alpha 1$  that was nearly equal

for both alcohols, indicating that there is no gain in potency upon lengthening of the decanol carbon chain. Thus, the alcohol cutoff is 10 for the Gly-R- $\alpha$ 1. Similar analyses of the GABA-R- $\rho$ 1 (Fig. 1*A Lower*) demonstrate a cutoff value of 7.

We next examined the alcohol cutoff of two chimeras used in our previous study (16). Chimera C1 (Fig. 1*B*) was composed of Gly  $\alpha$ 1 sequence from the N terminus of the receptor to the junction site in TM3 and GABA  $\rho$ 1 sequence thereafter to the C terminus. This chimera was gated by glycine, but not by GABA, and the effects of glycine on this chimera were enhanced by ethanol (16). The second chimera, C3 (Fig. 1*B*), was composed of GABA  $\rho$ 1 sequence from the N terminus to the junction site in TM1 and Gly  $\alpha$ 1 sequence thereafter to the C terminus. GABA, but not glycine, activated chimera C3, and the effects of GABA on this receptor were potentiated by ethanol (16). Longer-chain *n*-alcohols (e.g., hexanol or decanol) enhanced the actions of glycine or GABA on chimeras C1 and C3, respectively. As summarized in Fig. 1*B*, the alcohol cutoffs for both chimeras were found to be greater than  $n = 10$ . Because chimeras C1 and C3 have in common only a 63-amino acid region of the Gly  $\alpha$ 1 subunit located between the chimera junction sites in TM1 and TM3, these results indicate that the alcohol cutoff, at least for Gly-R- $\alpha$ 1 sequences, is determined by these 63 amino acid residues.

We next focused on two amino acid residues previously identified by site-directed mutagenesis of the Gly-R- $\alpha$ 1 (16) as crucial in mediating the effects of ethanol. These amino acids are located within TM2 (Ser-267) and TM3 (Ala-288), of the Gly-R- $\alpha$ 1 subunit. The homologous residues in the GABA-R- $\rho$ 1 are Ile-307 and Trp-328. We investigated the effects of ethanol and decanol on mutants at three of these positions as a screening assay for changes in the alcohol cutoff. Several amino acid substitutions at Ser-267 of the Gly-R- $\alpha$ 1 were made (17) and tested for ethanol (200 mM) and decanol (14  $\mu$ M) modulation of equieffective ( $EC_{5-10}$ ) concentrations of glycine. Decanol modulation of the glycine effect was decreased in some of these mutants, in comparison to its effect on the wild-type Gly-R- $\alpha$ 1. In particular, although ethanol had a substantial inhibitory effect on the mutant Gly-R- $\alpha$ 1 S267Q, as reported (17), decanol had no detectable effect, suggesting that this point mutation reduced the alcohol cutoff. This was verified directly by testing the effects of various concentrations of five *n*-alcohols on the glycine-gated currents of this mutant receptor (Fig. 2*A*). Several *n*-alcohols inhibited the glycine current in Gly-R- $\alpha$ 1 S267Q. Propanol was more potent than ethanol, whereas butanol was equally potent with propanol. Longer-chain *n*-alcohols (hexanol and octanol) were without effect on Gly-R- $\alpha$ 1 S267Q. Thus, for this mutant, the *n*-alcohol cutoff, defined as the point at which there is no further increase in potency, is  $n = 3$ . This represents a decrease in the cutoff of 7 carbon atoms, from a cutoff of  $n = 10$  in the wild-type Gly-R- $\alpha$ 1 receptor (Fig. 1*A*). In addition, this change in cutoff occurred without an apparent change in affinity for the agonist glycine; the  $EC_{10}$  concentrations of glycine for activation of the wild-type Gly-R- $\alpha$ 1 and the Gly-R- $\alpha$ 1 S267Q mutant were both 80–90  $\mu$ M. Similar results were obtained with the Gly-R- $\alpha$ 1 mutant S267Y; i.e., cutoff occurred at propanol (data not shown).

To determine whether the short alcohol cutoff of the GABA-R- $\rho$ 1 could be increased by mutation of the amino acid residues located at the homologous TM2 and TM3 positions of the GABA-R- $\rho$ 1, we made mutations of the GABA-R- $\rho$ 1 to the corresponding residue in the Gly-R- $\alpha$ 1, resulting in the GABA-R- $\rho$ 1 mutants I307S and W328A and the double mutant I307S/W328A. Ethanol inhibited the GABA-activated current in each of these mutants to an extent that was similar to that seen with the wild-type GABA-R- $\rho$ 1. However, the mutations altered the effects of long-chain alcohols: whereas decanol (14  $\mu$ M) had no significant effect on the GABA-activated current of the wild-type GABA-R- $\rho$ 1, this concen-

tration of decanol clearly potentiated the GABA effect on the I307S, W328A, and I307S/W328A GABA-R- $\rho$ 1 mutants. This potentiation was greatest ( $76 \pm 11\%$ ) with the I307S/W328A double mutant. The *n*-alcohol cutoff was investigated more closely in this GABA  $\rho$ 1 double mutant (Fig. 2*B*). Hexanol and the longer *n*-alcohols octanol, decanol, and dodecanol potentiated the GABA currents, with a potency that increased up to dodecanol, indicating that the alcohol cutoff for this mutant is dodecanol or higher [limited water solubility precluded testing of longer chain ( $n > 12$ ) alcohols]. Thus, substitution of bulky residues with smaller ones at positions 307 and 328 of the GABA-R- $\rho$ 1 increased the alcohol cutoff by at least 5 carbons, from  $n = 7$  in wild type to  $n \geq 12$  in the mutant. Again, this change in *n*-alcohol cutoff occurred without a change in apparent affinity of the receptor for the agonist GABA; for the wild-type GABA-R- $\rho$ 1 and GABA-R- $\rho$ 1 I307S/W328A double mutant, the  $EC_{10}$  concentrations of GABA were both 0.85–1.0  $\mu$ M. The alcohol cutoffs of the GABA  $\rho$ 1 receptor mutants containing single amino acid substitutions (I307S or W328A) were determined in a similar fashion and were found to be intermediate between that of the cutoff values for the wild-type and doubly mutated (I307S/W328A) GABA-R- $\rho$ 1 (Fig. 3).

## DISCUSSION

The original observations on the TM2 and TM3 point mutations in the glycine and GABA receptors (16) raised the crucial question of whether these residues represent a true binding site for the alcohols. In the present study, we found that alcohol cutoff in these receptors is determined solely by the TM2–TM3 region and that mutation of individual residues can decrease or increase the alcohol cutoff. The same mutations that elicit changes in alcohol cutoff do not significantly affect the apparent affinity of these LGICs for agonist or the functional gating of the LGICs, suggesting that the transduction of agonist binding energy into channel opening is unaltered in these mutants. The simplest explanation of these results is that the alcohols bind directly to the LGIC, outside of the ion-conducting pore but within a cavity formed between TM2 and TM3. An alternative explanation, which cannot be ruled out at this time, is that alcohols exert their effects at a hypothetical remote binding site, the dimensions of which are perturbed by substitutions within TM2 and TM3.

In this context, it is of interest to note that some of the mutations studied, such as the Gly-R- $\alpha$ 1 S267Q mutation, altered the effect of alcohols, from enhancing submaximal agonist responses to inhibiting agonist activity, in addition to reducing the alcohol cutoff. In this mutant receptor, we presume that the alcohols bind preferentially to the closed state of the ion channel (17). Conversely, the double mutation (I307S/W328) of the GABA-R- $\rho$ 1 not only increased the alcohol cutoff to accommodate decanol but also enabled decanol to enhance GABA responses, consistent with decanol binding to and stabilizing the open state of the channel. These hypotheses await further evaluation by using single channel recording techniques.

To attempt to visualize the effects of these point mutations on a putative alcohol binding cavity, the TM2 and TM3 domains of the wild-type and double mutant (I307S/W328A) GABA-R- $\rho$ 1 were modeled as antiparallel  $\alpha$  helices (Fig. 4). The true three-dimensional structure of these LGICs is unknown. These models are intended only as an aid to the estimation of the size of such a binding pocket and to suggest future experiments. In the case of TM2, an  $\alpha$ -helical structure is consistent with evidence for the homologous type A GABA receptor  $\alpha$ 1 subunit and for subunits of the nicotinic acetylcholine receptor (25, 26). By analogy with the work of Unwin (22) and Xu and Akabas (27), we have placed Ile-307 (analog of Ser-267 in the Gly-R- $\alpha$ 1) on the face of the helix away from

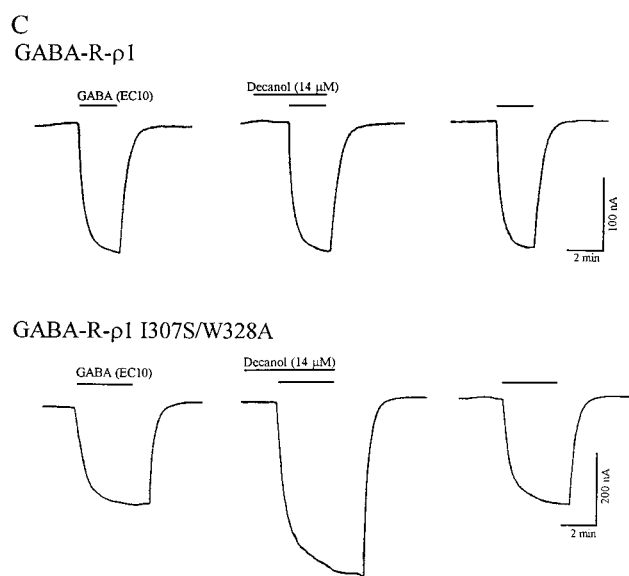
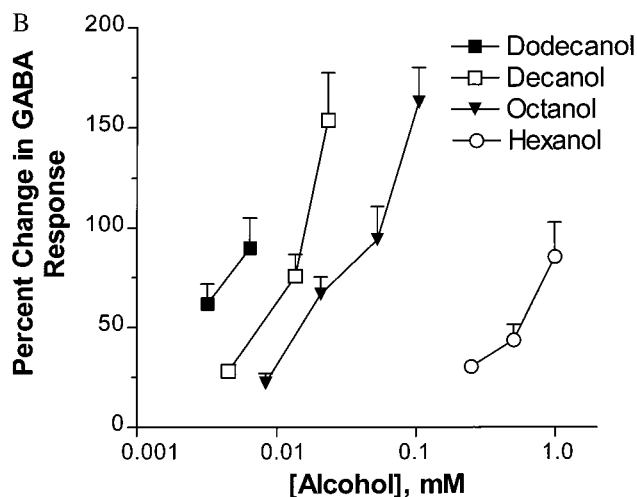
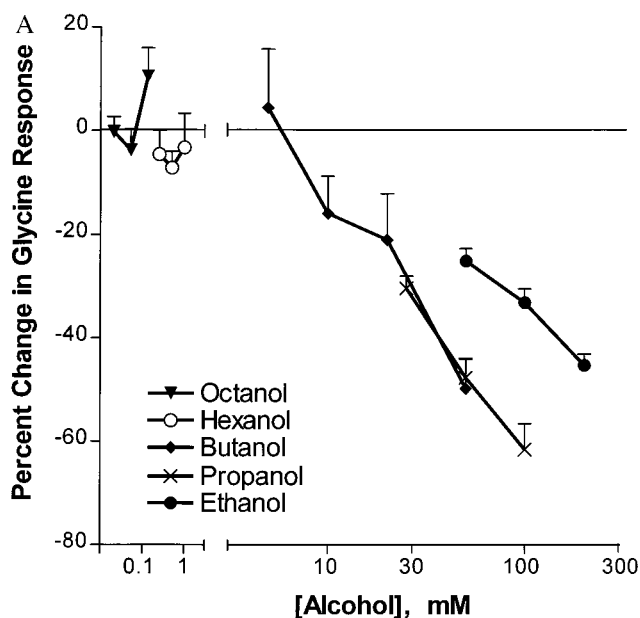


FIG. 2. (A) Determination of the *n*-alcohol cutoff of the Gly-R- $\alpha$ 1 S267Q mutant. Inhibition of the effects of an EC<sub>5</sub> concentration of glycine by various concentrations of methanol, ethanol, propanol, butanol, pentanol, hexanol, and octanol was determined in *Xenopus*

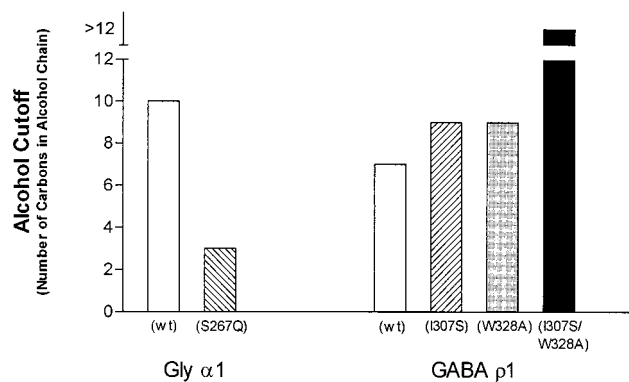


FIG. 3. Mutations of key amino acid residues of the Gly-R- $\alpha$ 1 and GABA-R- $\rho$ 1 alter the alcohol cutoff. Alcohol cutoff values for the wild-type Gly-R- $\alpha$ 1 and GABA-R- $\rho$ 1 are from Fig. 1A, and cutoff values for the Gly-R- $\alpha$ 1 S267Q and GABA-R- $\rho$ 1 I307S/W328A mutants are taken from Fig. 2A and B, respectively. Alcohol cutoff values for the GABA-R- $\rho$ 1 mutants I307S and W328A were determined as described in Fig. 2A and B. The bar representing the alcohol cutoff of the GABA-R- $\rho$ 1 I307S/W328A mutant (>12 carbons in the alcohol chain) is broken at 12 because greatly decreased aqueous solubility prevented testing of alcohols longer than dodecanol. All receptors were expressed in *Xenopus* oocytes.

the ion channel pore. There is no consensus for the secondary structure of TM3; for simplicity we have modeled this also as an  $\alpha$ -helix, consistent with evidence from one report (24). In this model, the simultaneous mutations of I307S and W328A create an enlarged "cavity" between TM2 and TM3 (Fig. 4B). It is possible to estimate the change in size of the proposed alcohol binding pocket caused by these mutations. By subtracting the volume of the serine and alanine residues from those of the larger isoleucine and tryptophan residues, we obtain an estimate of  $(53 + 125) \text{ \AA}^3 = 178 \text{ \AA}^3$ , assuming that no gross distortion of secondary structure occurs. Such a large increase in the size of the hypothetical cavity should allow longer-chain alcohols to be accommodated between the helices. But is the increase in size sufficient to account for the changes in cutoff observed? In the homologous series of *n*-alcohols, each additional methylene group adds approximately  $20 \text{ \AA}^3$  to the molecular volume. Therefore, these mutations should enable an increase in cutoff corresponding to an increase of 8 or 9 carbons in length. Because the observed change in cutoff was from 7 in the wild-type GABA-R- $\rho$ 1 to at least 12 in the GABA-R- $\rho$ 1 I307S/W328A mutant, we must conclude that the simplistic model presented herein provides some agreement between prediction and experimental data. For the mutations that decrease the alcohol cutoff of the Gly-R- $\alpha$ 1, we suggest the exact opposite mechanism, that amino acid residues (e.g., Gln and Tyr) that are larger than the

oocytes. Values shown are the percentage change in glycine response induced by the alcohols (mean  $\pm$  SEM, determined from 4 to 9 oocytes). Hexanol and octanol did not appreciably affect the glycine-gated responses of the Gly-R- $\alpha$ 1 S267Q mutant. (B) Determination of the *n*-alcohol cutoff of the GABA-R- $\rho$ 1 I307S/W328A double mutant, expressed in *Xenopus* oocytes. The effects of an EC<sub>10</sub> concentration of GABA were potentiated by various concentrations of hexanol, octanol, decanol, and dodecanol. Values represent the percentage change in GABA response induced by the alcohols (mean  $\pm$  SEM, determined from 5 to 10 oocytes). (C) Decanol potentiates the function of the doubly mutated GABA-R- $\rho$ 1 I307S/W328A receptor but not of the wild-type GABA-R- $\rho$ 1. Sample tracings of currents from voltage-clamped oocytes expressing the wild-type GABA-R- $\rho$ 1 (Upper) and GABA-R- $\rho$ 1 I307S/W328A (Lower). Currents were induced by application of an EC<sub>10</sub> concentration of GABA alone or GABA in the presence of 14  $\mu$ M decanol.

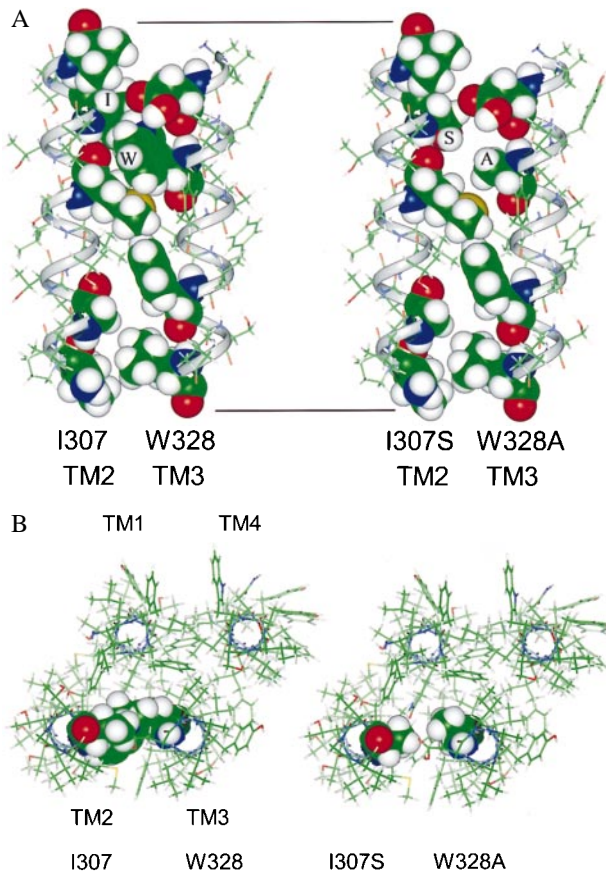


FIG. 4. (A) Molecular model of TM2 and TM3 of the human GABA  $\rho$ 1 receptor subunit. The wild-type TM domains are shown on the left; the I307S/W328A double mutant TM domains are shown on the right. The predicted TM2 was modeled as an  $\alpha$ -R-helix. However, in the assignment of the TM2 residues, those proposed by Unwin (22) for the nearly homologous TM2 of the nicotinic acetylcholine receptor (Val-308 to Val-325 of the GABA-R- $\rho$ 1) were used rather than residues Ala-303 to Val-325 of the GABA  $\rho$ 1 receptor proposed by Cutting *et al.* (23). The side of TM2 lining the ion channel pore is on the far left. TM3 was also modeled as an  $\alpha$ -helix. The axis of TM3 was aligned antiparallel with that of TM2 and then TM3 was rotated about its axis to obtain the closest approach of Ile-307 to Trp-328. Finally, the nonbonded interactions between the two helices were optimized. As shown on the right, the double mutation of the GABA  $\rho$ 1 receptor potentially creates a substantial cavity between the TM2 and TM3  $\alpha$ -helices. (B) A model of a GABA-R- $\rho$ 1 subunit viewed along the axis of the ion channel from the extracellular side. The dimer of TM2 and TM3 shown in A was used as a starting point for a model of a tetrameric subunit. TM2 was positioned so that the polar residues that line the ion channel face to the left. TM1 was built as an  $\alpha$ -helix (23) and aligned antiparallel with TM2 so that its axis was 11 Å from both TM2 and TM3 (an equilateral triangle). TM4 was built as an  $\alpha$ -helix and aligned antiparallel to TM3 so that its axis was 11 Å from TM3 and TM1. The backbone atoms of the four helices were tethered with a force constant of 10 kcal/Å (1 cal = 4.184 J). The positions of TM1 and TM4 were based on the data of Blanton and Cohen (24), demonstrating extent of exposure of the TMs of the acetylcholine receptor to hydrophobic photoactivatable probes: TM1 was only slightly labeled, TM3 was partially labeled, and TM4 was most labeled. The subunit on the left is wild-type GABA-R- $\rho$ 1 that has residues Ile-307 and Trp-328 shown with a van der Waals surface. The subunit on the right has the I307S and W328A mutations shown with a van der Waals surface.

native Ser effectively decrease the size of the alcohol binding pocket.

Although several recent studies have highlighted regions of LGICs that may be involved in alcohol action (16, 28, 29), the idea that alcohols exert their psychotropic actions by binding to discrete sites on membrane proteins has been slow to gain acceptance. In this context, it is of interest to consider another

system in which direct alcohol-protein interactions are known to take place. The metabolic enzyme alcohol dehydrogenase (ADH; EC 1.1.1.1) forms a substrate binding pocket for alcohols that is of finite size (30–32). ADH from yeast is highly selective for short-chain alcohols, whereas ADH from horse liver has a broader range of substrates. By mutating larger amino acid residues in the active site of yeast ADH to their smaller homologs, as are present in horse liver ADH, it was possible to broaden the substrate specificity of the yeast enzyme (33, 34). Structural data available on ADH suggest that these mutations increase the size of the substrate binding pocket in yeast ADH, thus allowing it to accept larger alcohol molecules. In our studies, modulation of the effect of a single concentration of decanol (14  $\mu$ M) by different amino acid substitutions at the Ser-267 position of the Gly-R- $\alpha$ 1 (11 substitutions in all) demonstrated that only the molecular volume of the substituted amino acid correlated, in a negative fashion, with the percent enhancement by decanol (data not shown). The measures of amino acid hydrophobicity, hydrophilicity, or polarity were not significantly correlated with the effect of decanol on the mutated Gly-R- $\alpha$ 1. In addition, Ye *et al.* (17) have recently shown that the effects of ethanol are modulated by amino acids substituted in the same position (Ser-267) on the Gly-R- $\alpha$ 1 and again the molecular volume of the substituted amino acids is correlated significantly with the effect of ethanol. The effects of amino acid substitutions within the alcohol binding pocket of ADH are clearly analogous to the present work; the results of this study on the LGICs suggest that manipulation of the alcohol cutoff in these receptors by mutagenesis reflects analogous changes in the physical size of a cavity or pocket. These data thus provide the strongest evidence to date in favor of an alcohol binding site on a central nervous system protein.

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