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Conformational Changes in the $GABA_A$ Receptor During Channel Gating and Alcohol Modulation

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Conformational Changes in the $GABA_A$ Receptor During Channel Gating and Alcohol Modulation

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Conformational Changes in the $GABA_A$ Receptor During Channel Gating and Alcohol Modulation

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The overall goal of this thesis is to structurally characterize the conformational change in the $GABA_A$ receptor during alcohol modulation. This thesis investigated which residues are changed in the TM3 region of the $GABA_A$ receptor $\alpha 1$ subunit during alcohol modulation. The substituted cysteine accessibility method has proven useful for investigating structural changes of the γ-aminobutyric acid type A ($GABA_A$) receptor during channel gating and allosteric modulation. In the present study, the surface accessibility and reaction rate of propyl- and hexyl-methanethiosulfonate (PMTS and HMTS) to cysteine residues introduced into the

third transmembrane segment (TM3) of the $GABA_A$ receptor α1 subunit were examined. GABA-induced currents in *Xenopus* oocytes expressing wild-type and cysteine-mutant $GABA_A$ receptors were recorded before and after application of MTS reagents in the resting, GABA- or alcohol (ethanol or hexanol) -bound states. The results indicate that a water-filled cavity exists around the A291 and Y294 residues of TM3, in agreement with previous results. Furthermore, the data suggest that a conformational change produced by alcohols (200mM ethanol or 0.5mM hexanol) exposure induces the water-cavity surrounding the A291C and Y294C residues to extend deeper, causing the A295C and F296C residues to become accessible to the MTS reagents. In addition, exposure of the A291C, Y294C, F296C and V297C mutants to MTS reagents in the presence of GABA had significant effects on their GABA-induced currents, indicating that the water-cavity around A291C and Y294C residues expanded to F296C and V297C by a structural movement due to GABA-binding. The data show that $GABA_A$ receptor is a dynamic protein during alcohol-modulation and channel-gating. Furthermore, mutations (A291W or S270I) of the critical sites of alcohol-binding pocket reduced significantly the accessibility of HMTS to A295C or F296C residues during alcohol modulation, suggesting that the A291 or S270 residues to alcohol-binding pocket are important sites for alcohol-induced conformational changes.

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Abbreviations

cRNA	complementary ribonucleic acid
$GABA_A$	γ-aminobutyric acid type A
nAChR	nicotinic acetylcholine receptor
SCAM	substituted cysteine accessibility method
LGIC	ligand-gated ion channel
DMSO	dimethyl sulfoxide
EC	effective concentration
MBS	modified Barth's solution
PMTS	propyl-methanethiosulfonate
HMTS	hexyl-methanethiosulfonate
NMDA	N-methyl-D-aspartate acid
$5\text{-}HT_3$	5-hydroxytryptamine type 3
pCMBS	*para*-chloromercuribenzene sulfonate
TM	transmembrane segment
WT	wild type

Chapter 1

Introduction

1.1 Acute alcohol effects on brain

Humans have been consuming alcohol for several thousands of years, and distillation of alcohol was developed by the Arabs about A.D. 800, and the word alcohol is derived from the Arabic for "something subtle" (Fleming et al., 2001). Following acute alcohol exposure, behavioral responses such as an increased reaction time, diminished fine motor control, impulsivity, or impaired judgment have been observed among humans, rodents, and fruit flies (Fraser and McAbee, 2004; Hansen-Trench and Barron, 2005; Hellemans et al., 2005; Wen et al., 2005). This indicates that alcohol disturbs the fine balance that exists between excitatory and inhibitory influences in the brain, resulting in the anxiolysis, ataxia and sedation (Fillmore and Weafer, 2004; Khisti et al., 2004; Moller et al., 1997; Newton et al., 2004; Spanagel et al., 1995). This can be accomplished by either enhancing inhibitory neurotransmission or antagonizing excitatory neurotransmission. Although alcohols have been long thought to act nonspecifically by some perturbation of cell membrane lipids in central nervous system (CNS) neurons, it is now believed that alcohols interact directly with proteins (Peoples et al., 1996). A number of results have shown that ethanol can affect the function of ligand-gated ion channels in the

brain, and ethanol is believed to produces its effects by simultaneously acting on multiple ion channel proteins that can affect neurotransmission (Allgaier, 2002; Harris, 1999; Lovinger, 1999; Narahashi et al., 1999), suggesting that there is a common ethanol-binding pocket shared by many of these proteins (Trudell and Harris, 2004).

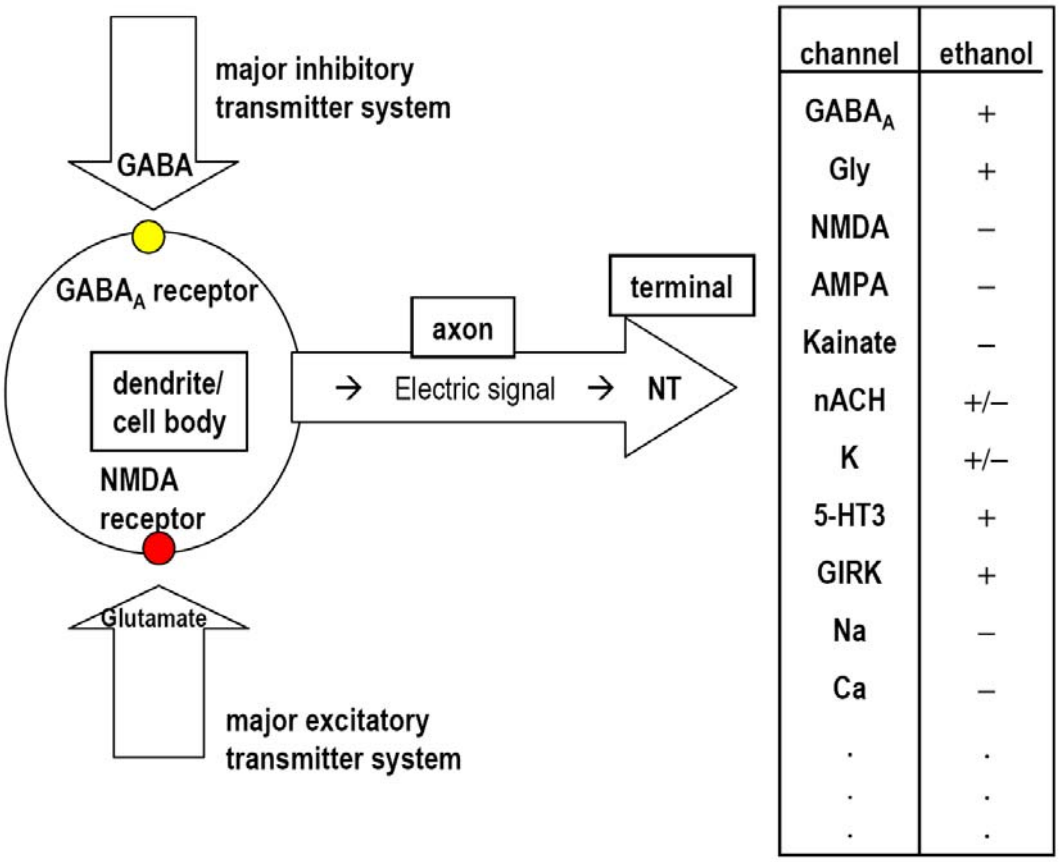

Figure 1. Schematic representation of alcohol-sensitive ion channels in the neuronal transmission of the brain. Alcohol (ethanol) enhances inhibitory synaptic transmission and antagonizes excitatory synaptic transmission by affecting many ion channels in the brain. +: excitatory effect of ethanol, -: inhibitory effect of ethanol

1.2 Alcohol-sensitive ligand-gated ion channels in the brain

A number of different types of ligand-gated ion channels, G protein-regulated channel proteins, and voltage-gated ion channels in the CNS are potential sites of alcohol action (Harris, 1999; Peoples et al., 1996) (Figure 1). The $GABA_A$ receptors mediate major inhibitory neurotransmission in the brain and have been also implicated as a major target of a number of classes of sedative, hypnotic, and anesthetic agents including barbiturates, benzodiazepines, and volatile anesthetics (Macdonald and Olsen, 1994; Mehta and Ticku, 1999). Furthermore, substantial behavioral, biochemical, and electrophysiological data indicate that the $GABA_A$ receptor act as an important target for the actions of alcohol (discussed further in 1.4 Acute Ethanol Effects on $GABA_A$ Receptors in this chapter).

Clinically relevant concentrations of ethanol have been also reported to potentiate the glycine receptor response in rat brain synaptoneurosomes (Engblom and Akerman, 1991), and cultured mouse hippocampal neurons (Aguayo and Pancetti, 1994).

Neuronal nicotinic acetylcholine receptors also may be prominent molecular target of alcohol action (Narahashi et al., 1999). Alcohols have been reported to produce both enhancement and inhibition of nicotinic acetylcholine receptor function, depending on the alcohol-chain length, concentration of alcohol tested, and recepter subunit combination (Peoples et al., 1996).

It has been shown that the function of the serotonin (5-hydroxytryptamine) 5-HT_3 receptor is potentiated in the presence of these alcohols at concentrations that are within the range of the blood and brain alcohol concentrations present during acute intoxication, suggesting that alcohol effects on the receptor contribute to intoxication (Lovinger, 1999).

Glutamate is the major excitatory neurotransmitter in the brain, and the excitatory ionotropic glutamate receptors are divided into the NMDA and non-NMDA receptors. Although the specific site of action of alcohol has not been identified on the NMDA receptor, the excitatory action of glutamate at NMDA receptors is inhibited by acute alcohol exposure (Allgaier, 2002). The non-NMDA receptors are subdivided into AMPA and kainate receptors. The majority of the studies performed on rat hippocampal neurons and expression systems, such as *Xenopus* oocytes and HEK293 cells, have shown that the function of AMPA and kainate receptors is inhibited by ethanol (Allgaier, 2002). Additionally, electrophysiological recording in motor neurons of rat spinal cord slices showed that ethanol directly inhibited AMPA and NMDA glutamate currents (Wang et al., 1999). Low concentrations of ethanol inhibit kainate receptors-mediated currents from electrophysiological recordings performed in CA1 pyramidal neurons and interneurons of rat hippocampal slices (Carta et al., 2003).

Although ligand-gated ion channels are a focus for specific molecular targets of ethanol action, many other types of channel proteins have been found to be

involved in action of alcohol. G-protein-gated inwardly rectifying K^+ (GIRK; Kir3) channels are involved in mediating slow postsynaptic inhibitory potentials, and loss of GIRK channels expressed in the brain results in hyperexcitability and seizures (Clancy et al., 2005). Function of GIRK channels was enhanced in the presence of pharmacologically relevant concentrations of ethanol, indicating that the GIRK channels may be important target sites for ethanol (Kobayashi et al., 1999; Lewohl et al., 1999). Some subtypes of voltage-gated Na(+) channels (Na(+) channels) were found to be inhibited by ethanol in the *Xenopus* oocyte expression system (Shiraishi and Harris, 2004). Interestingly, it was also reported that N-type calcium channels regulate acute responses to ethanol and ethanol reward and preference are modulated by the calcium channels in the mice (Newton et al., 2004). In addition, ethanol showed inhibitory effects on N- and T-type calcium channels in neural cells (Walter and Messing, 1999).

1.3 $GABA_A$ receptor

In the vertebrate CNS, γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter. GABA released from GABAergic neurons have been found to activate ionotrophic $GABA_A$ and $GABA_C$ receptors and metabotrophic $GABA_B$ receptors. Following GABA binding, the $GABA_A$ receptors directly gate channels and allow a passive flow of negatively charged chloride ions down their electrochemical gradient into neuron, thereby altering the membrane potential (Figure 2). Additionally, the $GABA_A$ receptors have modulatory binding sites for benzodiazepines, barbiturates, neurosteroids, anesthetics and alcohol. $GABA_B$ receptors are coupled to Ca^{2+} and K^+ channels via G proteins and second messenger systems (Bormann, 2000; Macdonald and Olsen, 1994). In this dissertation thesis, only $GABA_A$ receptors will be discussed.

The $GABA_A$ receptor is the major inhibitory neurotransmitter-gated and transmembrane heterooligomeric ion channel protein in the mammalian brain and is a member of the ligand-gated ion channel superfamily that includes the glycine, $GABA_C$ (GABA ρ), 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) and nicotinic acetylcholine (nACh) receptors (Bormann, 2000; Ortells and Lunt, 1995). The $GABA_A$ receptors are distributed very widely in the brain (Laurie et al., 1992a; Laurie et al., 1992b; Wisden et al., 1992)

1.3.1 $GABA_A$ receptor structure

Based on sequence similarity, several families and subtypes of $GABA_A$ receptor subunits (6 α, 3 β, 3 γ, 1 δ, 1 ε, 1 π and 1 θ subunits) have been cloned, identified and named (Bormann, 2000; Macdonald and Olsen, 1994; Whiting, 2003). About 30-40% sequence identity exists among subunit families and all of the sequences within each subunit family indicates about 70-80% amino acid sequence homology (Macdonald and Olsen, 1994). Additional variety is obtained from the existence of several splice variants, such as α6, β2 and γ2 subtypes (Korpi et al., 2002; Korpi et al., 1994; Macdonald and Olsen, 1994; Taylor et al., 2000). Recently, novel ionotropic GABA receptors that are composed of ρ-subunits have been defined in the vertebrate retinal neuron and have been also classified as $GABA_C$ receptors, which are picrotoxin ($GABA_A$ receptor-antagonist)-sensitive. The ρ 1–3 subunits of $GABA_C$ receptors show similar structural characteristics and sequence homology as the other $GABA_A$ receptor subunits (Bormann, 2000; Korpi et al., 2002).

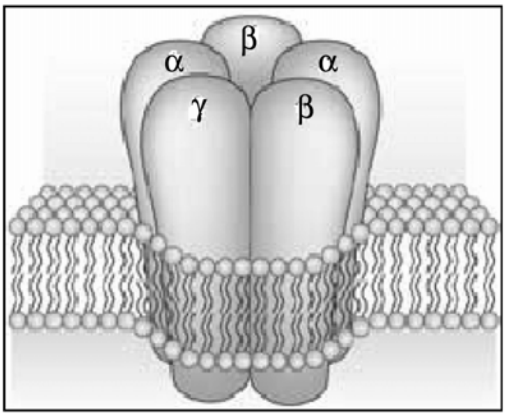

Figure 2. Overview of the $GABA_A$ receptor in the cell membrane. $GABA_A$ receptors span the lipid bilayer of cell membrane and are composed of five subunits surrounding a central chloride-ion channel.

The $GABA_A$ receptor is arranged pseudosymmetrically around a central pore formed by mainly the second transmembrane domain (TM2) of five homologous subunits and a heteropentameric glycoprotein of about 275 kD (Macdonald and Olsen, 1994; Ortells and Lunt, 1995). Analysis of the amino acids sequences of all known $GABA_A$ receptor subunits suggest that each subunit of about 50 kD has a large N-terminus extracellular domain with cysteine loop and putative N-glycosylation sites, four transmembrane domains (TM1, TM2, TM3, and TM4), a large intracellular loop between TM3 and TM4, and a short extracellular C terminus (Figure 3). The agonist-binding site has been primarily localized to the N-terminal domains of the α and β subunits. The intracellular loop of some subunits contains the highly variable sequence involved in cytosolic regulatory mechanisms such as phosphorylation by protein kinase A and C or tyrosine protein kinase (Amin and Weiss, 1993; Macdonald and Olsen, 1994; Schofield et al., 1987; Sigel et al., 1992; Smith and Olsen, 1994).

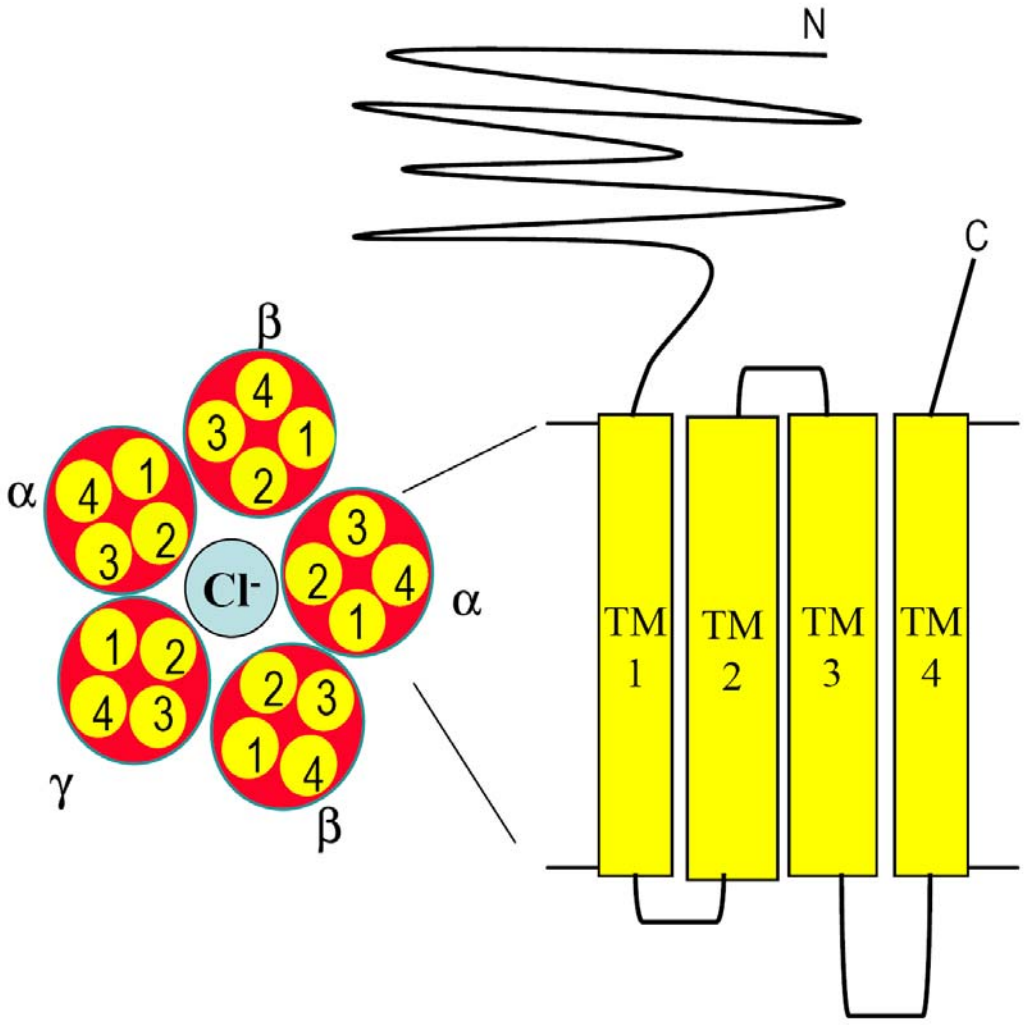

Figure 3. Model of the $GABA_A$ receptor in the cell membrane. Most common $GABA_A$ receptor is composed of five subunits, which are two α, two β and one γ subunits. Four putative membrane-spanning helices are shown as cylinders. The interfaces of extracellular N-terminal domains from a and b subunits have presumed to have putative GABA-binding pockets . A large putative intracellular loop between TM3 and TM4 segments is proposed to have phosphorylation sites. There is also a extracellualr C-terminal domain at the end of TM 4 region.

Functional $GABA_A$ receptors can be formed from only α and β subunits in the heterologous expression systems. However, the most prevalent stoichiometry and subtype of $GABA_A$ receptors in the brain are believed to be 2 α, 2 β and 1 γ subunits (Chang et al., 1996; Farrar et al., 1999; Korpi et al., 2002; Laurie et al., 1992a; Laurie et al., 1992b; Tretter et al., 1997; Wisden et al., 1992).

1.3.2 GABA-binding and channel-opening

In the pentameric complex of $GABA_A$ receptor, it has been suggested that there are two putative GABA-binding pockets at the interfaces of extracellular N-terminal domains from α and β subunits. Amino acids that participate in ligand binding at N-terminal domains of α and β subunits have been identified by photoaffinity labeling and sequencing, as well as by site-directed mutagenesis (Amin and Weiss, 1993; Hartvig et al., 2000; Korpi et al., 2002; Sigel et al., 1992; Smith and Olsen, 1994; Wagner and Czajkowski, 2001). To explain coupling processes of agonist binding to opening of the ion channel, it has been proposed that binding of ligand to the N-terminal agonist-binding domain triggers a small rotation of the extracellular domains of the receptor subunits. It was also suggested that an extracelluar structural change produced by binding of agonist to the N-terminal region might be transferred to TM regions possibly through the TM2-3 linker to open

the channel (Kash et al., 2003; Lynch et al., 2001). Recently, conformational changes by these rotations of binding-site subunits following agonist binding have been suggested to be communicated into the adjoining TM2 regions, and the rotations induce a rotation of the TM2 segments which then opens the channel pore formed by the adjoining TM2 regions in the homologous nACh receptor (Miyazawa et al., 2003; Unwin, 2005).

1.4 Acute ethanol effects on $GABA_A$ receptors

Several neurotransmitter receptors and ion channels in brain have been suggested as sites for alcohol action (Harris, 1999; Peoples et al., 1996). The $GABA_A$ receptor, which mediates the majority of inhibitory synaptic transmissions in the brain, has been implicated as an important target of alcohols and anesthetics (Deitrich et al., 1989; Harris, 1999; Nestoros, 1980).

A number of studies testing actions of agonists, antagonists or inverse agonists of $GABA_A$ receptor have showed that the pharmacological and behavioral effects of ethanol are mediated via central $GABA_A$ receptors. Ro15-4513 (imidazobenzodiazepine), which is a partial benzodiazepine receptor inverse agonist (indirect $GABA_A$ receptor antagonist), reversed the intoxicating properties of ethanol in mice and rats (Hoffman et al., 1987; Suzdak et al., 1986a; Suzdak et al., 1988).

Picrotoxin, $GABA_A$ receptor antagonist, antagonized the anxiolytic effects of ethanol (Becker and Anton, 1990; Glowa et al., 1988). Ethanol-induced impairment of the aerial righting reflex was stimulated and antagonized by microinjection of the $GABA_A$ receptor agonist, muscimol and $GABA_A$ receptor antagonist, bicuculline, respectively, into the medial septal area (Givens and Breese, 1990).

Physiologically relevant concentrations of ethanol (20-60 mM) induced potentiation of muscimol- or GABA-mediated $^{36}Cl^-$-uptake by early biochemical studies in mice spinal cord cultured neurons neurons and microsacs derived from rat and mouse cerebral cortex (Allan and Harris, 1986; Engblom and Akerman, 1991; Mehta and Ticku, 1988; Suzdak et al., 1986b). Furthermore, clinically relevant concentrations of ethanol potentiated GABA-activate currents in mouse hippocampal neurons (Aguayo and Pancetti, 1994; Reynolds et al., 1992), cultured rat cerebellar Purkinje cells (Sapp and Yeh, 1998), cultured embryonic rat cortical neurons (Marszalec et al., 1998), and chick cerebral cortical neurons (Reynolds and Prasad, 1991). Additionally, in the heterologous systems (*Xenopus* oocytes and mammalian cells) expressing $GABA_A$ receptor, ethanol has also shown to potentiate $GABA_A$ receptor-mediated chloride currents (Harris et al., 1995; Mihic et al., 1994; Sigel et al., 1993; Ueno et al., 2000; Ueno et al., 1999b; Wallner et al., 2003; Whitten et al., 1996).

Interestingly, the subunit composition of the $GABA_A$ receptor can alter the effect of ethanol. Clinically relevant concentrations of ethanol potentiated

pharmacologically isolated $GABA_A$ receptor-mediated currents recorded from hippocampal CA1 neurons of rat brain slice. Interestingly, the ethanol-mediated enhancement of $GABA_A$ receptor- mediated synaptic transmission required intracellular ATP and was blocked by specific inhibitors of protein kinase C (PKC), indicating that PKC-mediated phosphorylation can modulate ethanol sensitivity of pharmacologically isolated $GABA_A$-mediated synaptic current (Weiner et al., 1994). There are two splice variants of the $GABA_A$ receptor γ2 subunit, which are original short γ2S variant and larger γ2L variant containing additional eight amino acids with a unique phosphorylation site for PKC in its putative intracellular loop between TM3 and TM4 (Whiting et al., 1990). The γ2L variant has been also found to be involved in potentiation of recombinant $GABA_A$ receptors by low concentrations of ethanol (Harris et al., 1995; Wafford and Whiting, 1992). Furthermore, δ subunit-containing $GABA_A$ receptors have been shown to exist outside the synapse and ambient extracellular GABA concentrations (0.5-1μM) can activate the αβδ-$GABA_A$ receptors (Mody, 2001). Interestingly, recent studies have showed that δ subunit-containing $GABA_A$ receptors (expressed in *Xenopus* oocytes) are sensitive to low concentrations of ethanol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), suggesting that extrasynaptic $GABA_A$ receptors may be primary targets for ethanol underlying low-dose alcohol intoxication (Hanchar et al., 2005).

Ethanol and most volatile anaesthetics (*e.g.* isoflurane) enhance the functions of $GABA_A$ and glycine receptors, whereas these drugs inhibit the GABA ρ1 receptor.

Analysis of sequence alignments and chimeric receptor constructs identified two specific amino-acid residues, Ser270 and Ala291, (Ser267 and Ala 288, homologous residues at the equivalent positions of the glycine receptor α1 subunit) in TM2 and TM3 of $GABA_A$ receptor α1 subunit that are required for actions of ethanol and some volatile anesthetics (Mihic et al., 1997). Additionally, other site-directed mutagenesis studies have suggested that these two S270/A291 residues, and equivalent S267/A288 residues are critical sites of alcohol-binding pocket in the $GABA_A$ and glycine receptors (Mascia et al., 2000b; Wick et al., 1998).

1.5 SCAM and the MTS reaction

The substituted cysteine accessibility method (SCAM) has been used to investigate structure and conformational changes of ion channel domains in different functional states (Boileau et al., 2002; Karlin and Akabas, 1998; Lobo et al., 2004a; Lynch et al., 2001; Williams and Akabas, 1999; Williams and Akabas, 2000; Williams and Akabas, 2002). To investigate structural dynamics of extracellular binding sites or transmembrane region (TM) of ion channel during gating and modulation, a single cysteine residue is introduced into the fuctional domain. The mutants containing cysteine substitution are expressed heterologously. If the functional properties of the mutant channels are similar to wild type, it is possible to test the ability of water-soluble, sulfhydryl-specific reagents to react with the engineered cysteines. A major assumption of SCAM is that the substitution of cysteine for a candidate amino acid does not disrupt the assembly and structure of the channel protein radically. For many cases, mutation to cysteine is very well tolerated and the agonist concentration-response curves of the cysteine mutants are not altered radically by the mutation (Lobo et al., 2004a; Lynch et al., 2001; Newell and Czajkowski, 2003; Pascual and Karlin, 1998; Teissere and Czajkowski, 2001; Wagner and Czajkowski, 2001; Williams and Akabas, 1999; Williams and Akabas, 2000; Williams and Akabas, 2001; Williams and Akabas, 2002). In the TM region of channel protein, the substituted cysteine is located in the water-accessible surface, in the lipid-accessible surface or in inside of the protein and probed state-dependently

(in the resting or any functional states) with a water-soluble, sulfhydryl-specific reagent. With SCAM, it is assumed that the cysteine residues that react with MTS reagents are exposed at a water-accessible protein surface because water is required to ionize cysteine residues. MTS reagents are sulfhydryl-specific compounds and react with ionized thiolate groups ($-S^-$) 10^9 times faster than with un-ionized thiols (-SH) (Karlin and Akabas, 1998; Roberts et al., 1986) (Figure 4). Previous results have indicated that the subset of cysteine-substitution mutants that react with sulfhydryl-specific reagents are markers for the specific conformational states induced by different ligands. Furthermore, if the substituted cysteine reacts with the reagent to cause the function of the channel to be irreversibly changed, the cysteine is assumed to be exposed at the water-accessible protein surface in the specific functional state (Boileau et al., 2002; Karlin and Akabas, 1998; Lobo et al., 2004a; Lynch et al., 2001; Newell and Czajkowski, 2003; Pascual and Karlin, 1998; Teissere and Czajkowski, 2001; Wagner and Czajkowski, 2001; Williams and Akabas, 1999; Williams and Akabas, 2000; Williams and Akabas, 2001; Williams and Akabas, 2002).

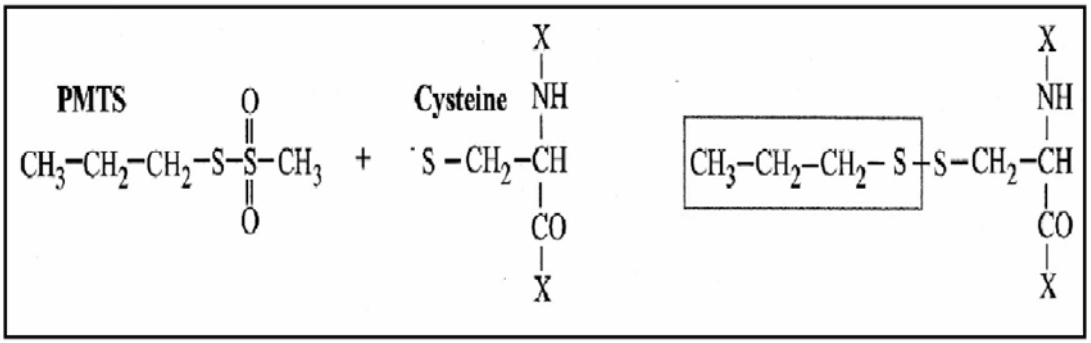

Figure 4. Propyl-MTS (methanethiosulfonate) is an example of sufhydryl specific compounds (propyl- or hexyl-MTS). It is shown above to covalently react with the thiolate group (aqueous environment) of a cysteine substituted on the target protein. In the case of this study, this is a Cys residue introduced in the TM 3 region of $GABA_A$ receptor $\alpha 1$ subunit.

1.6 Specific aims

The overall experimental goal is to structurally characterize the conformational change in the $GABA_A$ receptor during alcohol modulation. Particularly, these experiments will define which residues are changed in the TM3 region of the $GABA_A$ receptor $\alpha 1$ subunit during alcohol modulation by using SCAM. The structural changes of the water-filled cavity at the upper region in the TM3 region of the $GABA_A$ receptor $\alpha 1$ subunit produced by actions of alcohol may provide a molecular basis for allosteric modulation of $GABA_A$ receptors by alcohols. This study includes 3 specific aims.

Aim 1. To determine whether alcohols induce conformational changes in $GABA_A$ receptor $\alpha 1$ subunit, as determined by reactivity (or accessibility) of MTS reagents to each amino acid of TM 3 domain,

Aim 2. To investigate whether alcohol-induced conformational changes are different from conformational changes induced by GABA,

Aim 3. To determine whether mutations of critical sites (S270 or A291) of alcohol-binding pocket affect alcohol-induced conformational changes using double mutants (*e.g.*, S270I/A295C).

1.7 Chapter overview

Chapter 2 describes experimental methods used for these specific aims. These include site-directed mutagenesis, isolation and injection of *Xenopus* oocytes, electrophysiological two-electrode voltage-clamp recording, substituted accessibility method (SCAM) experiments and statistical analyses used.

Chapter 3 focuses on a question raised from Aim 1. It is known that an alcohol-binding pocket exists between TM2 and TM3 regions in the $GABA_A$ $\alpha1$ subunit. There is also evidence that the pocket is a water-filled cavity and the structure of this cavity is altered with agonist and modulators. We propose that the conformational changes produced by alcohols will also alter this cavity. More specifically, it was investigated whether alcohols produce conformational changes in TM3 and some of these changes are also similar to ones produced by GABA or other modulators, but others are unique to the alcohols. Using the substituted cysteine accessibility method (SCAM), it was investigated whether residues in the TM3 segment become water-accessible as consequence of conformational change, assuming that if cysteine mutant is reactive to MTS reagents, the position of cysteine is water-accessible. Mutants where each residue in the TM3 domain of the $GABA_A$ receptor $\alpha1$ subunit was individually mutated to cysteine were tested. The wild-type or mutant $\alpha1$ subunits were expressed with the wild-type $\beta2$ and $\gamma2s$ $GABA_A$ receptor subunits in *Xenopus* oocytes, and the effects of these mutations were

investigated using two-electrode voltage-clamp recording. The ability of each mutant to react with the sulfhydryl-specific reagents, propyl- or hexyl-methanethiosulfonate (PMTS or HMTS) state-dependently in the presence of alcohol or GABA and in the resting state were investigated.

Chapter 4 focuses on questions raised in Aim 2 of the dissertation thesis. The chapter 3 shows that A291C, Y294C, A295C, F296C or V297C mutants were reactive to MTS reagents in the resting-, alcohol- or GABA-bound states. It is suggested that reaction-rate of MTS to accessible Cys-mutants depends on the collision frequency between MTS reagents and thiolate (-S-) group of cysteine introduced. This collision frequency indicates a local microenvironment of the accessible cysteine. The micoenvironments are further characterized by physical environment of cysteine and ionization of thiol group to thiolate group. Physical environment explains how open space is around the cysteine residue, the ionization indicates that how many water molecules are there around the cysteine residue.

Reaction rates of these reactive mutants to investigate microenvironments of around these residues in the various functional states were tested and compared.

Chapter 5 focuses on Aim 3 of the dissertation proposal. Chapters 3 and 4 show that A295C and F296C mutants were accessible to hexyl-methanethiosulfonate (HMTS) in the alcohol-bound states, not in the resting state. This indicates that A295C and F296C sites became water-accessible by alcohol-induced conformational changes following alcohol binding. Previous data also show that S270 of TM2 and

A291 of TM3 are critical sites of alcohol-binding pocket. It was tested whether A295C or F296C mutants containing S270I or A291W mutations (double mutants ; S270I/A295C, S270I/F296C, A291W/A295C and A291W/F296C) become accessible to HMTS in the presence of alcohols to support that S270 and A291 are important sites for alcohol action.

Chapter 6 describes an overall discussion of these results obtained and conclusions derived

Chapter 2

Materials and Methods

2.1 Site-directed mutagenesis and cRNA synthesis

pGEMHE plasmids encoding wild type and 16 cysteine mutants from A291 to V307 in TM3 of the rat $GABA_A$ α1 subunit were kind gifts from Akabas group (Albert Einstein college of medicine, Yeshiva University, New York) and described previously (Williams and Akabas, 1999). One additional single mutant (I290C) also made by using pGEMHE plasmid encoding the wild-type rat GABA receptor α1 subunit using the Quik-change site directed mutagenesis kit (Stratagene, LaJolla, CA) (Figure 5). To make double mutants (S270I/A295C and S270I/F296C or A291W/A295C and A291W/F296C), the second desired substitution-mutations (S270I or A291W) were introduced into pGEMHE plasmids encoding the rat $GABA_A$ receptor α1 (A295C) or (F296C) subunits using the same Quik-change site directed mutagenesis kit. All mutagenic sense and antisense primers for each substitution-mutation were designed and ordered from Integrated DNA Technologies, Inc. (Coralville, IA). All these site-directed mutations were verified by DNA sequencing performed at Core Sequencing Facility of The University of Texas at Austin. cRNAs were synthesized from pCDMA8 plasmid encoding human β2 $GABA_A$ receptor subunit and pGEMHE plasmids encoding wild-type/mutant rat α1

or wild-type γ2s $GABA_A$ receptor subunits by using T7 RNA polymerase kit (Stratagene, LaJolla, CA).

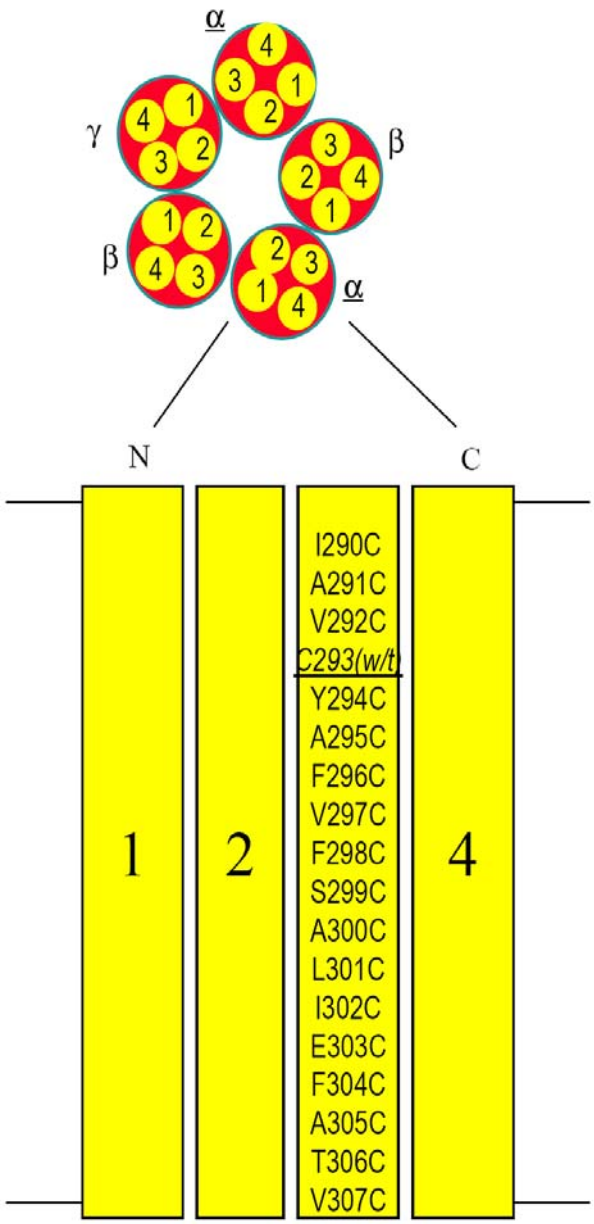

Figure 5. Schematic representation of positions of wild type and 17 cysteine substitution mutations from I290 to V307 in TM3 of the rat $GABA_A$ α1 subunit. Four putative membrane-spanning helices are shown as cylinders from a subunit. The top side shows extracellular end, and bottom side shows intracellular end. The each 17 Cys mutant and wild type $GABA_A$ receptors were expressed in separate oocytes individually.

2.2 Isolating *Xenopus laevis* oocytes and injecting cRNAs

Frog oocytes were harvested from *Xenopus laevis* (African clawed frogs, NASCO International, Fort Atkinson, WI and Xenopus Express, Plant City, FL). The frogs were kept in tanks on a 12-hr light/dark cycle at 19°C and fed a diet of fishmeal or frog brittle two times per week. Before surgery to harvest oocytes, a mature female frog was first anesthesized and a small incision through the skin and muscle layers on one side of the abdomen was made by using sterile scissors and sterile forceps. Then, a ovarian tissue was removed and placed in a dish of oocyte culture medium. After suturing the muscle wall and then the skin, the frog was allowed to recover and returned the colony. In order to isolate oocytes from the ovarian tissue, ovarian lobes were place in hypertonic isolation media (108 mM NaCl, 2mM KCl, 1mM EDTA and 10mM HEPES 10, pH 7.5). The function of this media makes the oocytes to shrink from the encapsulating membranes, which allows the membranes to be isolated from oocytes. Stage V and VI oocytes were dissected with fine surgical forceps and placed in sterile filtered incubation media (modified Barth's solution, MBS containing 10 mg/liter streptomycin, 50 mg/liter gentamicin, 10,000 units penicillin/liter, 90 mg/l theophylline and 220 mg/l pyruvate; MBS, 88 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91mM $CaCl_2$, pH 7.5). Dissected oocytes were incubated for 10 min in buffer containing 0.5 mg/ml collagenase Type IA and 83mM NaCl, 2mM

KCl, 1mM $MgCl_2$ and 10mM HEPES 10, pH 7.5, to remove the follicular cell layer and then the oocytes were again placed in the incubation media (Dildy-Mayfield and Harris, 1992). For injections, cRNAs (10ng/50nl) were injected into the animal/vegetal boundary of each oocyte by using a microdispenser (Drummond Scientific, Broomwall, PA). The cRNAs encoding wild-type or mutant α1, wild-type β2 and γ2s subunits combinations in a 1:1:1 ratio were stored in diethylpyrocarbonate (DEPC)-treated water (Williams and Akabas, 1999). For single mutants, cRNAs of αl subunits encoding I290C, A291C, V292C, Y294C, A295C, F296C, V297C, F298C, S299C, A300C, L301C, I302C, E303C, F304, A305C or T306C, and for double mutants, cRNAs of αl subunits encoding S270I/A295C, S290I/F296C, A291W/A295C or A291W/F296C were co-injected with cRNAs encoding wild-type β2 and γ2s subunits. The injecting glass needles were pulled with a flaming/brown micropipette puller (Sutter Instrument Co., Novato, CA) and the tips of needles cut to a diameter of 10-20 μm. The injected oocytes were stored in incubation media in Corning 96 cell wells (Corning Glass Works, Corning, NY) at 13°C.

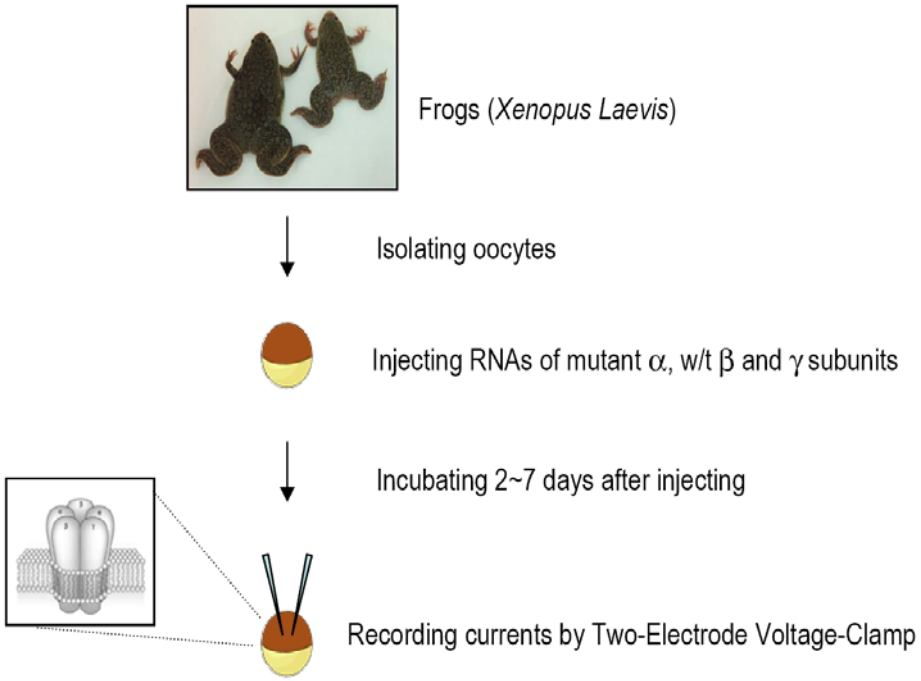

Figure 6. Isolation and injection of *Xenopus* oocytes. Oocytes were isolated from *Xenopus* frogs, injected with cRNAs encoding αβγ subunits , and then GABA-induced currents were measured using two-electrode voltage clamp electrophysiology.

2.3 Electrophysiological recording

GABA-induced currents were recorded from oocytes 2-7 days after cRNA injection using a two-electrode voltage-clamp (Mascia et al., 2000a) (Figure 6). All measurements were made at room temperature (21-23°C). Oocytes were placed in a 100 µl volume rectangular chamber and perfused at 1.8 ml/min with MBS. The perfusion rate was maintained by using peristaltic pump (Cole-Parmer Instruments Co., Chicago, IL). 18-gauge polyethylene tubing system (Becton Dickinson, Sparks, MD) was used for flow of MBS in and out of the bath. The animal pole of oocytes was impaled with two glass electrodes (1.0-5.0 megaohm) filled with 3 M KCl. Oocytes were voltage-clamped at -70 mV using an oocyte clamp OC725C (Warner Instruments, Hamden, CT). Clamping currents were plotted on a chart recorder (Cole-Parmer Instrument, Vernon Hills, IL).

2.4 GABA concentration-response curves

GABA concentration-response curves were determined from individual oocytes expressing wild-type or mutant $GABA_A$ receptors. Various concentrations of GABA (0.1µM-1mM) were prepared in MBS and applied for 25 sec. First, 1mM GABA (maximal GABA) was applied, next GABA solutions in order of increasing

concentration were applied sequentially to the oocytes. Finally, 1mM GABA solution was again applied after a plateau of GABA-induced responses was obtained. In all cases, the oocytes were washed out for 5-10 min with only MBS after each application of GABA. Each GABA-induced response was normalized to the averaged maximal current in each oocyte, and fitted by nonlinear regression analysis (see below 2.8 Data Analysis).

2.5 Percentage potentiation of GABA-induced currents by alcohols

A maximal current was obtained with 1mM GABA and EC_{5-10} was determined for each oocyte. To determine potentiation of the GABA EC_{5-10}, which is concentration of agonist that evoke 5-10% of the maximal current, response by alcohols (200 mM ethanol or 0.5 mM hexanol), oocytes were perfused with alcohols for 1 min to allow for complete equilibration before a 25 sec coapplication with GABA (EC_{5-10}). In all cases, oocytes were washed out for 10-15 min after application of the alcohol/GABA solutions. The solutions were freshly prepared immediately before use. Percentage potentiation of the GABA response by the alcohols was then calculated as the percentage increase above the control (EC_{5-10}) response to GABA in the presence of ethanol or hexanol.

2.6 The reaction of MTS reagents to Cys mutants

2.6.1 Accessibility of MTS reagents to Cys mutants

To investigate the surface accessibility of MTS reagents to $GABA_A$ receptors, GABA control currents were first determined using two different GABA concentrations corresponding to the EC_{5-10} and EC_{50}. These currents were determined to be stable if the change was <5% on more than two consecutive applications. Second, oocytes expressing wild-type or mutant $GABA_A$ receptors were perfused 90 sec with PMTS or HMTS solutions in the resting state (0.5 mM MTS reagents alone), GABA-bound state (0.5 mM MTS reagents in 1 mM GABA) or alcohol-bound states (0.5 mM MTS reagents in 200 mM ethanol or 0.5 mM hexanol). Experiments testing different functional-states were performed in the separate oocytes. Following washing (10 ~ 15 min) with MBS, the GABA control currents (EC_{5-10} & EC_{50}) were redetermined. The effect of MTS reagents was calculated as follows: The % change = $\{I_{after}/I_{before}) - 1\} \times 100$, where I_{before} and I_{after} indicate the values of the two control currents induced by EC_{5-10} or EC_{50} GABA concentrations before and after the application of the MTS reagent.

2.6.2 Measurement of reaction rates

MTS reaction rates with the introduced cysteines were determined by the effect of sequential brief applications of MTS in the resting, alcohol-bound, or GABA-bound states (Bera et al., 2002; Lobo et al., 2004a; Wagner and Czajkowski, 2001). After stabilization of EC_{5-10} or EC_{50} GABA-induced currents on more than two consecutive applications, HMTS alone (for resting state), HMTS + 1 mM GABA (for GABA-bound state) or HMTS + 200 mM ethanol or 0.5 mM hexanol (for alcohol-bound states) solutions were applied for 20 sec; 0.25 - 0.5 mM concentrations of HMTS were used. All MTS and alcohol solutions were prepared immediately before use. After washing (10 - 15 min) with MBS, the EC_{5-10} and EC_{50} GABA-induced currents were remeasured. To determine the effects of alcohols on reaction rates in alcohol-bound states, 200 mM ethanol or 0.5 mM hexanol solutions in MBS were preincubated for 60 sec, as for the alcohol-potentiation experiments. This procedure was repeated until the GABA-induced response no longer changed. The normalized GABA-induced currents to the initial current were plotted as a function of the cumulative duration of HMTS application and fitted to one phase exponential function using GraphPad Prism3 software (San Diego, CA) to calculate the first order rate constants (Bera et al., 2002; Lobo et al., 2004a). To determine the second order rate constant ($M^{-1}s^{-1}$), the first order rate constants obtained from the

single exponential fitting were divided by the concentration of MTS used (Pascual and Karlin, 1998).

2.7 Chemicals and Reagents

GABA, ethanol (100%) and hexanol (98%) were obtained from Sigma-Aldrich (St. Louis, MO). MTS reagents (PMTS and HMTS) were obtained from Toronto Research Chemicals (North York, ON, Canada). Solutions of GABA, ethanol or hexanol were prepared in MBS. Stock solutions of PMTS and HMTS were first prepared in dimethyl sulfoxide (DMSO) and then diluted in MBS to a final DMSO concentration not exceeding 0.05%. In all experiments, the solutions of alcohols or MTS reagents were freshly prepared immediately before use.

2.8 Data Analysis

All values are presented as mean $\pm$ standard error of the mean (S.E.) from 4 or more independent experiments. Nonlinear regression analysis was performed to determine EC_{50} and Hill coefficient values from GABA concentration-response curves by using GraphPad Prism3 software (San Diego, CA). The data were

normalized to the averaged maximal current in each oocyte, and fitted according to the equation of the form: $I = I_{max}/[1 + (EC_{50}/A)^n]$, where I is current, I_{max} is the maximal current recorded in a given oocyte, EC_{50} is the GABA concentration for half-maximal current response, A is the GABA concentration, and n is the Hill coefficient.

In the experiments determining accessibility of MTS reagents to Cys mutants, statistical significance of the difference between each mutant and wild type was analyzed by one way analysis of variance (ANOVA) with the Dunnett's posthoc test with $P < 0.05$ representing significance using GraphPad Prism3 software (San Diego, CA).

Chapter 3

Accessibility of MTS Reagents to Each Cysteine Mutant of $GABA_A$ Receptor α1 Subunit in the Various Functional States

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Jung S, Akabas MH and Harris RA (2005) Functional and structural analysis of the $GABA_A$ receptor α1 subunit during channel gating and alcohol modulation. *J Biol Chem* 280(1):308-16.

3.1 Introduction

Previous SCAM results have suggested that channel gating and allosteric modulation by diazepam or propofol induce conformation changes in the TM3 of $GABA_A$ receptor α1 subunit, and that water-accessible cavities form around the TM3 segment. This indicates that structural movement by GABA-, diazepam- or propofol-binding might allow sulfhydryl-specific reagents to penetrate into the interior of the TM domain (Williams and Akabas, 1999; Williams and Akabas, 2000; Williams and Akabas, 2002). Recently, it was shown that the conformational changes accompanying channel gating increase accessibility of MTS reagents to amino acids critical for actions of alcohol and volital anestheics in TM1, TM2, and TM3 regions of the homologous glycine receptors (Lobo et al., 2004a). It has been known that several amino acids (S270/A291 from $GABA_A$ receptor and S267/A288 from glycine receptor) in the TM2 and TM3 segments are critical for potentiating $GABA_A$ and glycine receptors by volatile anesthetics and alcohols (Mihic et al., 1997). A binding cavity for alcohols may exist in a crevice near the extracellular ends of the TM2 or TM3 regions of $GABA_A$ and glycine receptors (Mascia et al., 2000a; Mascia et al., 2000b; Wick et al., 1998). Furthermore, it was demonstrated that the S267 and A288 amino acids in the TM2 and TM3 segments of glycine receptor α subunit are close and facing each other (Lobo et al., 2004c). However, the structure and function of the TM3 of $GABA_A$ receptor during alcohol-binding

remains unknown. In the present study, we asked how the structure of the alcohol-bound $GABA_A$ receptor differs from ones in the resting or GABA-bound states.

The SCAM was used to investigate the structure and conformational changes of the TM3 region of $GABA_A$ receptor $\alpha 1$ subunit during channel gating and alcohol modulation. It is proposed that a water-filled cavity exists around the extracellular side of TM3, and that the cavity deepens to the middle region of TM3 during channel gating and alcohol-binding. Furthermore, based on results of functional accessibility of PMTS and HMTS, our data suggest that the extracellular side of TM3 is more flexible or dynamic compared to cytosolic side of TM3. Thus, structural rearrangement of the existing water-filled cavities around the extracelluar side of TM3 during channel gating and alcohol-binding may have an important role in both forming the binding sites for alcohols and in the mechanism of allosteric modulation of the $GABA_A$ receptor by alcohols.

3.2 Expression and functional characterization of wild-type and cysteine mutants

$GABA_A$ receptors composed of only α and β subunits are able to bind GABA or alcohols and transduce their effects in the recombinant system (Mihic et al., 1997; Ueno et al., 2000). However, the αβγ-subunit (particularly $\alpha_1\beta_2\gamma_2$) composition of $GABA_A$ receptor are the prevalent combination in the mammalian brain (Farrar et al., 1999; Ortells and Lunt, 1995). Therefore, the $\alpha_1\beta_2\gamma_{2S}$-combination for wild-type and mutant $GABA_A$ receptors expressed in *Xenopus* oocytes was used in this study. To assess whether cysteine mutations affected $GABA_A$ receptor function, each individual cysteine mutant α1 subunits were coexpressed with wild-type β_2 and γ_{2S} subunits in oocytes. Then, the GABA-induced currents (I_{GABA}) and alcohol potentiation of I_{GABA} were determined (Tables 1 and 2).

Table 1. GABA EC_{50}, Hill coefficients and maximum currents for the wild type and mutant $GABA_A$ receptors in oocyte

mutant	EC_{50} (μM)	Hill coefficient	I_{max} (nA)
$\alpha_1\beta_2\gamma_{2s}$	44.9±4.6	1.34±0.07	3954±435
α_1(I290C)$\beta_2\gamma_{2s}$	53.5±2.0	1.27+0.12	1793±172
α_1(A291C)$\beta_2\gamma_{2s}$	35.8±3.5	1.14±0.12	1443±342
α_1(V292C)$\beta_2\gamma_{2s}$	54.7±7.3	1.38±0.11	3294±435
α_1(Y294C)$\beta_2\gamma_{2s}$	11.6±3.4	1.17±0.16	1700±275
α_1(A295C)$\beta_2\gamma_{2s}$	123±10	1.50±0.17	1406±160
α_1(F296C)$\beta_2\gamma_{2s}$	111±10	1.26±0.11	1180±327
α_1(V297C)$\beta_2\gamma_{2s}$	95.9±18	1.15±0.24	3250±327
α_1(F298C)$\beta_2\gamma_{2s}$	117±4.3	1.45±0.10	1333±153
α_1(S299C)$\beta_2\gamma_{2s}$	27.9±6.4	0.94±0.09	2523±329
α_1(L301C)$\beta_2\gamma_{2s}$	208±22	1.74±0.09	820±75
α_1(I302C)$\beta_2\gamma_{2s}$	32.9±11	1.44±0.51	2594±363
α_1(E303C)$\beta_2\gamma_{2s}$	24.4±1.8	1.03±0.08	1851±254
α_1(F304C)$\beta_2\gamma_{2s}$	17.2±3.2	1.34±0.40	1736±397
α_1(A305C)$\beta_2\gamma_{2s}$	23.8±3.3	1.41±0.23	1614±112
α_1(T306C)$\beta_2\gamma_{2s}$	96.2±12	1.49±0.10	2438±413
α_1(V307C)$\beta_2\gamma_{2s}$	78.1±8.6	1.43±0.09	2730±456

Values are presented as mean ± S.E. from 4 to 8 oocytes (A300C receptor is not included because of little current response to GABA).

Most of the mutants showed GABA-induced currents similar to wild type, except for the A300C mutant, which showed little current response to 1 mM GABA (<200 nA). In general, cysteine substitutions were well tolerated within the region I290-V307 of TM3. The mutations produced some changes in GABA sensitivity, with the most sensitive (Y294C) and the least sensitive (L301C) having GABA EC_{50} values about 4-fold different from wild-type receptors. For wild-type receptors, 200 mM ethanol or 0.5 mM hexanol potentiated GABA EC_{5-10}-induced currents by 66 $\pm$ 5% and 70 $\pm$ 7%, respectively (Table 2). These concentrations approximately correspond to the anesthetic concentration *in vivo* (Alifimoff et al., 1989; Fang et al., 1997). Ethanol and hexanol altered GABA EC_{5-10}-induced currents of all other cysteine mutants by amounts ranging from -8 $\pm$ 4 to 67 $\pm$ 8% and 35 $\pm$ 4 to 93 $\pm$ 4%, respectively (Table 2).

Table 2. Percentage potentiation of currents induced by GABA (EC_{5-10}) by 200mM ethanol or 0.5mM hexanol for wild type and mutant $GABA_A$ receptors.

mutant	ethanol (200mM)	hexanol (0.5mM)
$\alpha_1\beta_2\gamma_{2s}$	67±5.3	70±2.9
α_1(I290C)$\beta_2\gamma_{2s}$	58±8.5	71±9.8
α_1(A291C)$\beta_2\gamma_{2s}$	13±4.2	47±3.3
α_1(V292C)$\beta_2\gamma_{2s}$	52±5.0	71±3.2
α_1(Y294C)$\beta_2\gamma_{2s}$	-8±1.7	54±5.9
α_1(A295C)$\beta_2\gamma_{2s}$	20±3.8	75±14.4
α_1(F296C)$\beta_2\gamma_{2s}$	20±1.1	55±4.5
α_1(V297C)$\beta_2\gamma_{2s}$	67±8.1	72±3.2
α_1(F298C)$\beta_2\gamma_{2s}$	23±6.4	93±3.7
α_1(S299C)$\beta_2\gamma_{2s}$	17±3.1	35±4.3
α_1(L301C)$\beta_2\gamma_{2s}$	23±6.4	82±2.5
α_1(I302C)$\beta_2\gamma_{2s}$	22±2.4	59±7.6
α_1(E303C)$\beta_2\gamma_{2s}$	47±2.9	75±2.3
α_1(F304C)$\beta_2\gamma_{2s}$	63±9.0	75±10.0
α_1(A305C)$\beta_2\gamma_{2s}$	36±2.1	63±7.6
α_1(T306C)$\beta_2\gamma_{2s}$	59±8.0	84±3.3
α_1(V307C)$\beta_2\gamma_{2s}$	60±8.3	90±8.8

Values are presented as mean ± S.E. from 5 to 8 oocytes (A300C receptor is not included because of little current response to GABA).

3.3 Reaction of introduced cysteines with MTS reagents in the resting state

Next, the surface accessibility of uncharged propyl- and hexyl-methanethiosulfonate (PMTS and HMTS) as sulfhydryl-specific reagents to covalently label cysteines introduced into the TM3 domain was examined. The wild-type or mutant α_1 subunits were co-expressed with wild-type β_2 and γ_{2S} subunits in *Xenopus* oocytes. For PMTS and HMTS, GABA-induced currents in *Xenopus* oocytes expressing wild-type $GABA_A$ receptors before and after treatment with MTS compounds were first determined. Exposure of wild-type $GABA_A$ receptors to MTS compounds had no significant effects on GABA-induced currents (Figures 7 and 8). Additionally, application (1.5 min) of MTS compounds in the presence of GABA (1 mM), ethanol (200 mM) or hexanol (0.5 mM) did not affect GABA-induced currents in wild-type $GABA_A$ receptor (Figures 7, 8 and 9). Thus, endogenous cysteine residues were inaccessible for reaction with the MTS compounds, or reaction with the MTS compounds had no functional effects in wild-type $GABA_A$ receptors under these conditions (resting, GABA- or alcohol-bound states). Next, whether conformational changes induced by agonist (GABA) or modulators (ethanol or hexanol) in the mutant receptors alter the accessibility of MTS reagents, PMTS and HMTS, to introduced cysteine in TM3 was investigated. First, GABA-induced currents were recorded before and after treatment with MTS reagents without GABA or alcohols (resting state). The GABA-induced currents from A291C and Y294C

mutants receptors were significantly changed after applying MTS reagents to these mutant receptors in the resting state (Figures 7, 8 and 9).

Interestingly, the Y294C mutant showed opposite results from PMTS and HMTS treatments: PMTS inhibited GABA-induced currents, but HMTS potentiated GABA-induced currents at Y294C (Figures 7, 8 and 9). The results from A291C and Y294C mutants were consistent with previous findings that a water-pocket exists around these amino acids (Mascia et al., 2000b; Williams and Akabas, 1999). Application of MTS reagents in the resting states did not affect the function of any other receptors (Figures 7 and 8). This implies that these mutant receptors in the resting states are not accessible to MTS reagents, or reaction of the MTS reagents had no significant effects on mutant receptors.

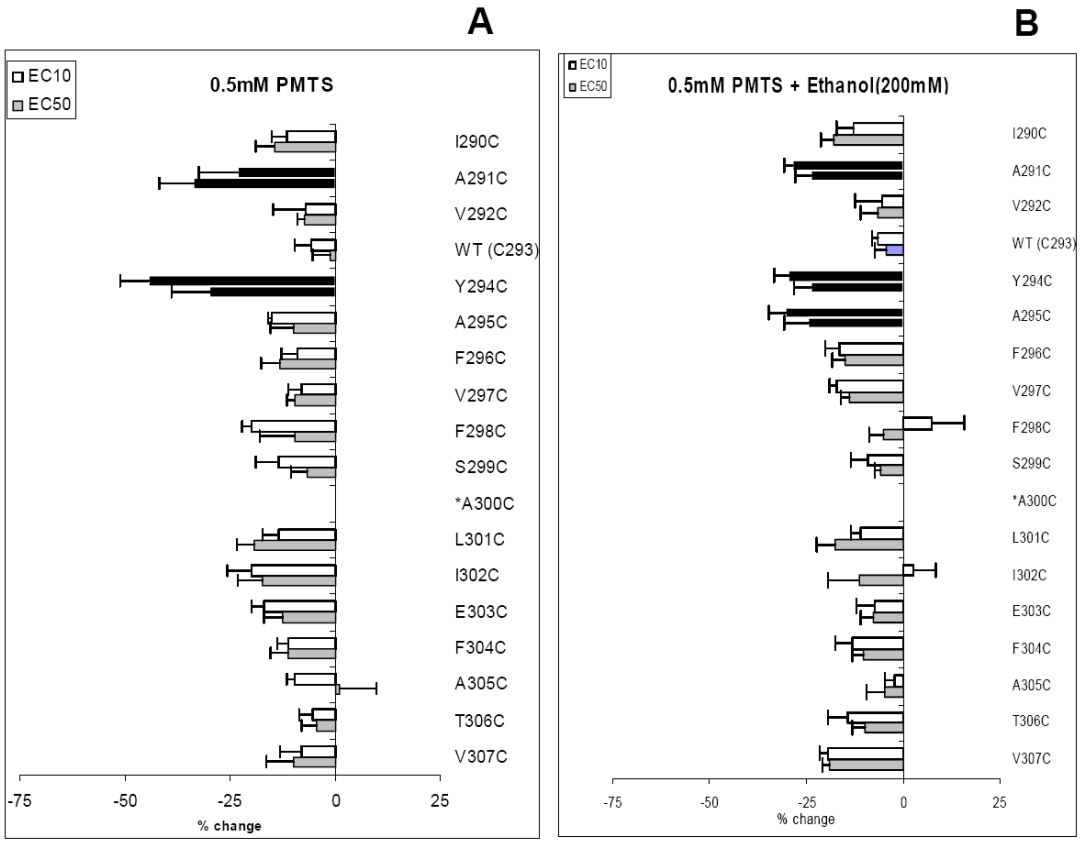


Figure 7. Effects of PMTS on GABA-induced currents of wild-type and mutants $GABA_A$ receptors in the resting state (A), or ethanol-bound state (B). For GABA-induced currents, the GABA $EC_{5\sim10}$ (top bar, white) and EC_{50} (lower bar, gray) were applied on wild-type and mutant $GABA_A$ receptors. Black bars indicate effects that are significantly different statistically from the effect on wild type in each condition by a one-way ANOVA, using the Dunnet post hoc test. The % change was calculated as = $\{I_{after}/I_{before}) - 1\} \times 100$, where I_{before} and I_{after} indicate the values of the two GABA-induced currents before and after the application of the sulfhydryl reagent (0.5 mM applied for 90 sec). All values are presented as mean $\pm$ S.E. from 3 to 8 oocytes. *A300C receptor is not included because of little current response to GABA.

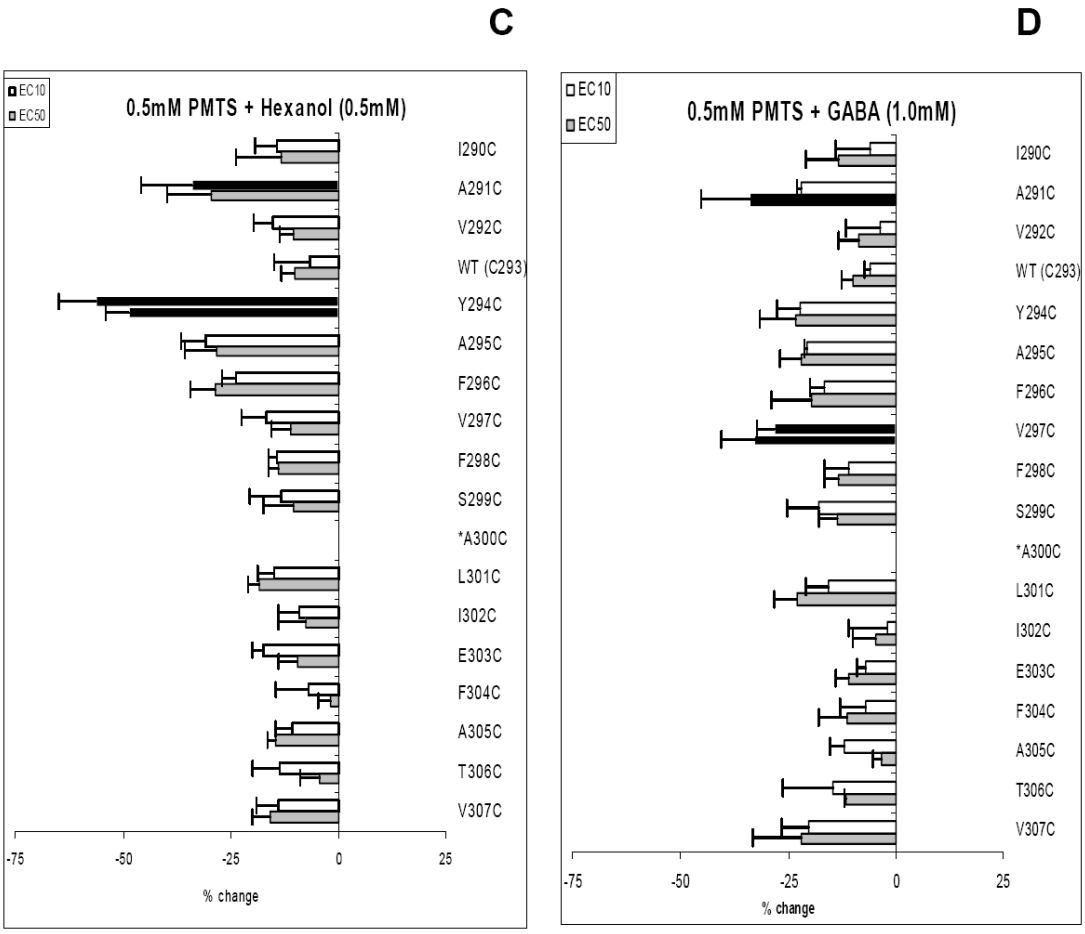


Figure 7. Effects of PMTS on GABA-induced currents of wild-type and mutants $GABA_A$ receptors in the hexanol-bound state (C) or GABA-bound state (D). For GABA-induced currents, the GABA $EC_{5\sim10}$ (top bar, white) and EC_{50} (lower bar, gray) were applied on wild-type and mutant $GABA_A$ receptors. Black bars indicate effects that are significantly different statistically from the effect on wild type in each condition by a one-way ANOVA, using the Dunnet post hoc test. The % change was calculated as = $\{I_{after}/I_{before}) - 1\} \times 100$, where I_{before} and I_{after} indicate the values of the two GABA-induced currents before and after the application of the sulfhydryl reagent (0.5 mM applied for 90 sec). All values are presented as mean $\pm$ S.E. from 3 to 8 oocytes. *A300C receptor is not included because of little current response to GABA.

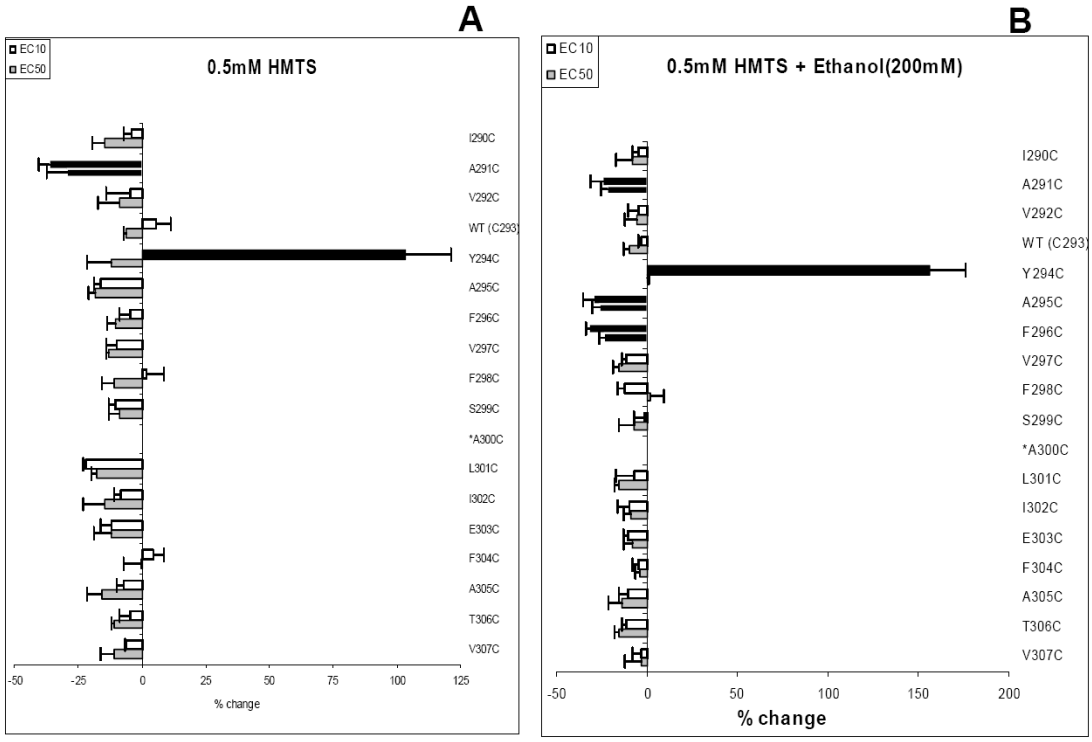


Figure 8. Effects of HMTS on GABA-induced currents of wild-type and mutants $GABA_A$ receptors in the resting state (A), or ethanol-bound state (B). For GABA-induced currents, the GABA $EC_{5\sim10}$ (top bar, white) and EC_{50} (lower bar, gray) were applied on wild-type and mutant $GABA_A$ receptors. Black bars indicate effects that are significantly different statistically from the effect on wild type in each condition by a one-way ANOVA, using the Dunnet post hoc test. The % change was calculated as = $\{I_{after}/I_{before}) - 1\} \times 100$, where I_{before} and I_{after} indicate the values of the two GABA-induced currents before and after the application of the sulfhydryl reagent (0.5 mM applied for 90 sec). All values are presented as mean $\pm$ S.E. from 3 to 8 oocytes. *A300C receptor is not included because of little current response to GABA.

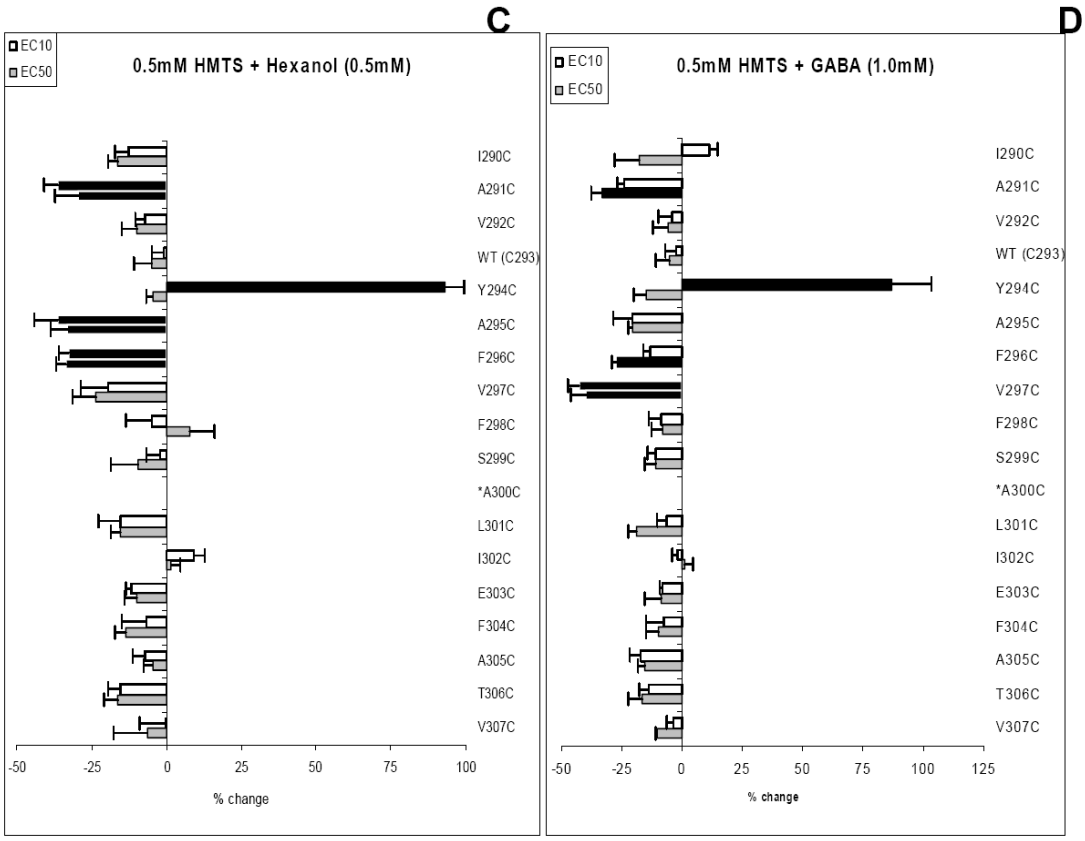


Figure 8. Effects of HMTS on GABA-induced currents of wild-type and mutants $GABA_A$ receptors in the hexanol-bound state (C) or GABA-bound state (D). For GABA-induced currents, the GABA $EC_{5\sim10}$ (top bar, white) and EC_{50} (lower bar, gray) were applied on wild-type and mutant $GABA_A$ receptors. Black bars indicate effects that are significantly different statistically from the effect on wild type in each condition by a one-way ANOVA, using the Dunnet post hoc test. The % change was calculated as = $\{I_{after}/I_{before}) - 1\} \times 100$, where I_{before} and I_{after} indicate the values of the two GABA-induced currents before and after the application of the sulfhydryl reagent (0.5 mM applied for 90 sec). All values are presented as mean $\pm$ S.E. from 3 to 8 oocytes. *A300C receptor is not included because of little current response to GABA.

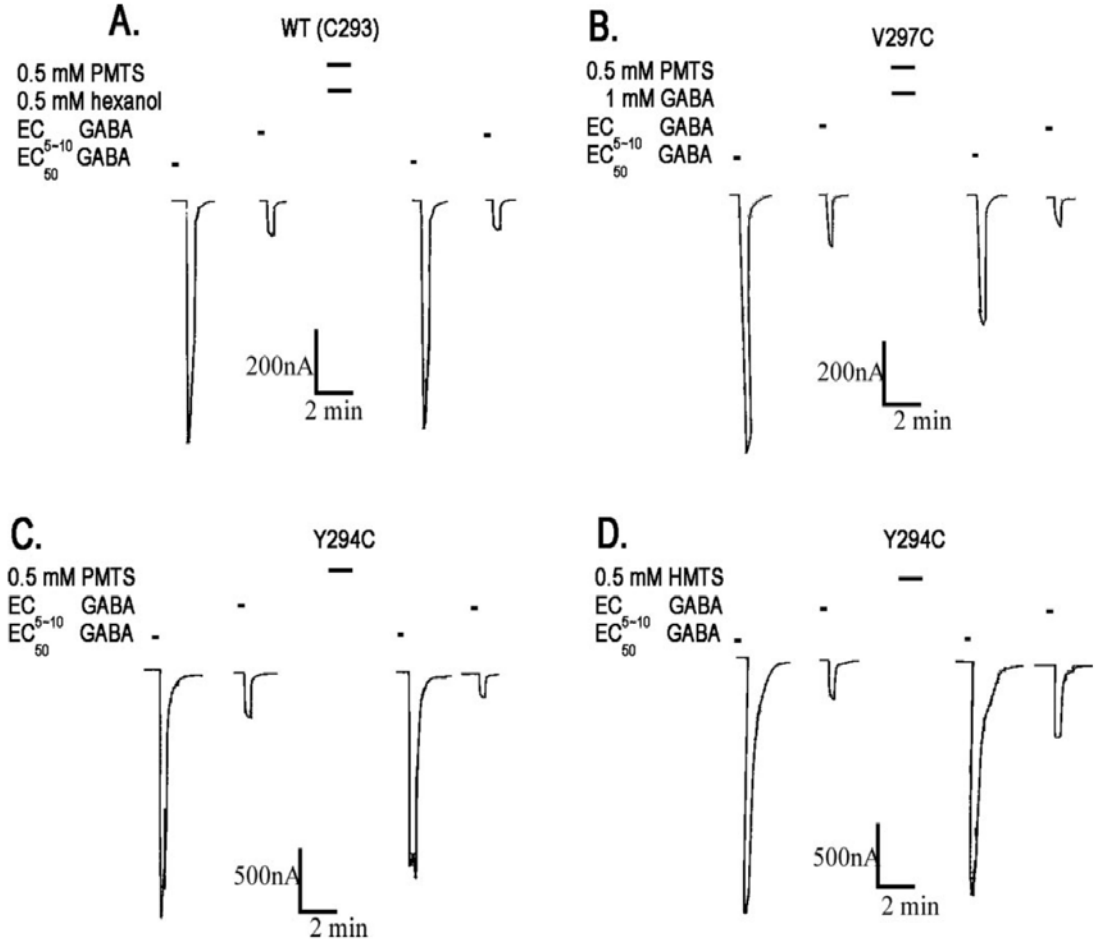


Figure 9. Effects of MTS reagents on wild-type and mutant $GABA_A$ receptors. Sample tracings obtained from oocytes expressing wild-type (C293) (A), V297C (B), Y294C (C, D) $GABA_A$ receptors demonstrating the effects of MTS solutions (0.5 mM) of various conditions on currents induced by GABA (EC_{5-10} or EC_{50}). (A) no effect of PMTS applied in the hexanol-bound state, (B) inhibitory effect of PMTS applied in the GABA-bound state, (C) inhibitory effect of PMTS applied in the resting state, and (D) potentiating effect of HMTS applied in the resting state.

3.4 Reaction of introduced cysteines with MTS reagents in the GABA-bound state

Next, whether application of MTS reagents in the presence of GABA (1mM) affects GABA-induced currents of the mutant $GABA_A$ receptors was investigated. Application of MTS reagents with 1mM GABA significantly changed the GABA EC_{5-10}- or EC_{50}-induced currents of the A291C, Y294C, F296C and V297C mutants (Figures 7 and 8). Y294C or F296C mutants had no significant effects of PMTS in the GABA-bound state, but showed significant effects of HMTS in the GABA-bound state (Figures 7 and 8). The A291C and Y294C mutants were also affected by MTS reagents in the resting state. Treatment with PMTS or HMTS in the presence of GABA significantly inhibited GABA-induced currents at A291C, F296C and V297C mutants (Figures 7, 8 and 9). Results of the Y294C mutant were similar to results obtained in the resting state. PMTS with GABA also inhibited GABA-induced currents, but HMTS with GABA potentiated GABA-induced currents at Y294C mutant (Figures 7 and 8). The other mutants were not significantly affected by MTS reagents in this condition.

3.5 Reaction of introduced cysteines with MTS reagents in the alcohol-bound states

Finally, GABA-induced currents in oocytes expressing mutant $GABA_A$ receptors were determined before and after treatment of MTS reagents with ethanol or hexanol to test if there are changes of accessibility of MTS reagents in the alcohol-bound state. GABA-induced currents of A291C, Y294C, A295C and F296C mutants receptors were significantly changed after applying MTS reagents with alcohols (Figures 7 and 8). A295C or F296C mutants had no significant effects of PMTS in the ethanol- or hexanol-bound states, but showed significant effects of HMTS in the ethanol- and hexanol-bound states (Figures 7 and 8). MTS reagents applied with alcohols had no significant effect on all other mutants tested (Figures 7 and 8). A291C and Y294C mutants were already reactive to MTS reagents in the resting and GABA-bound states. Treatment with PMTS or HMTS in the presence of ethanol or hexanol significantly inhibited GABA-induced currents at A291C, A295C and F296C mutants. For the Y294C mutant, PMTS with ethanol or hexanol also inhibited GABA-induced currents, and HMTS with ethanol or hexanol potentiated the currents (Figures 7 and 8).

3.6 DISCUSSION

3.6.1 Accessibility of MTS reagents and structural rearrangements

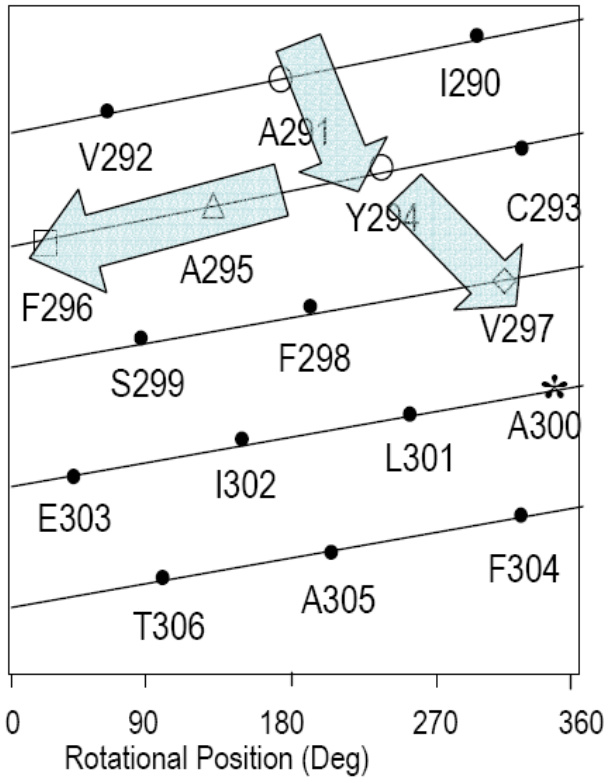
With SCAM, it is assumed that the cysteine residues that react with MTS reagents are exposed at a water-accessible protein surface because water is required to ionize cysteine residues. MTS reagents react with ionized thiolate groups ($-S^-$) 10^9 times faster than with un-ionized thiols (-SH) (Karlin and Akabas, 1998; Roberts et al., 1986). Previous results have indicated that the subset of cysteine-substitution mutants that react with sulfhydryl-specific reagents are markers for the specific conformational states induced by different ligands (Lobo et al., 2004a; Lynch et al., 2001; Newell and Czajkowski, 2003; Pascual and Karlin, 1998; Teissere and Czajkowski, 2001; Wagner and Czajkowski, 2001; Williams and Akabas, 1999; Williams and Akabas, 2000; Williams and Akabas, 2001; Williams and Akabas, 2002). In particular, using SCAM it was shown that TM3 of the $GABA_A$ receptor $\alpha 1$ subunit undergoes conformational changes during channel gating and allosteric benzodiazepine- or propofol-modulation (Williams and Akabas, 2000; Williams and Akabas, 2002). Alcohols are also assumed to produce allosteric modulation of $GABA_A$ receptors, but nothing is known about conformational changes produced by alcohols and possible overlap with changes produced by GABA, benzodiazepines and propofol. In this study, the SCAM was used to investigate whether there are common conformational changes in TM3 of the $GABA_A$ receptor $\alpha 1$ subunit during

alcohol-binding and GABA-binding. In this study, it is assumed that the mutant is accessible to the MTS reagent if either of GABA EC_{5-10} or EC_{50}-induced currents are affected after treatment by an MTS reagent. It has been found that the A291C and Y294C mutants in the resting state were significantly accessible to MTS reagents, consistent with these amino acids at the extracellular end of TM3 being on the water-accessible protein surface or facing a water-filled cavity as was proposed earlier (Mascia et al., 2000a; Williams and Akabas, 1999). It has been suggested that this water-filled cavity is connected to the extracellular solution, at least transiently allowing water and MTS reagents to enter and that residues lining the cavity can therefore react with applied MTS reagents in the resting state (Williams and Akabas, 2002). MTS reagents also reacted with the A291C, Y294C A295C and F296C mutants in the presence of alcohols. The results imply that an alcohol-induced conformational change may induce the water-filled cavity around A291C and Y294C to extend deeper, causing the A295C and F296C residues to be accessible to MTS reagents (Figures 10).

Another possibility is that alcohol-binding could induce the formation of a separate water-filled cavity around A295C or F296C that is transiently connected to the extracellular solution to allow PMTS or HMTS to reach these residues. In this case the cavity around A291C/Y294C may not be connected to the one that forms in the alcohol-bound state around A295C/F296C. Given the proximity of these residues, however, this seems unlikely. Furthermore, exposure of the A291C, Y294C, F296C

and V297C mutants to MTS reagents in the presence of GABA had significant effects on their GABA-induced currents, indicating that the water-accessible surface around A291C and Y294C residues widened to include F296C and V297C due to a structural movement induced by GABA-binding (Figure 10). It is also possible that residue V297 simply becomes accessible by distortion of this local environment in the presence of GABA. However, V297 is located below Y294 in our model (Figure 10), therefore, it is likely that V297C becomes accessible to MTS reagents by expansion of the cavity containing water and MTS reagents in the GABA-bound state. These data also support the idea that an extracelluar structural change produced by binding of GABA to the N-terminal region must be transferred to this TM3 region possibly through the TM2-3 linker (Kash et al., 2003; Lynch et al., 2001). The A291C and Y294C mutants are accessible to MTS reagents in the resting state and also in the alcohol-bound and in the GABA-bound states.

A. α-helical net projection



○, reactive with MTS in the resting-, alcohol- and GABA-bound states

△, reactive with MTS in the alcohol-bound states

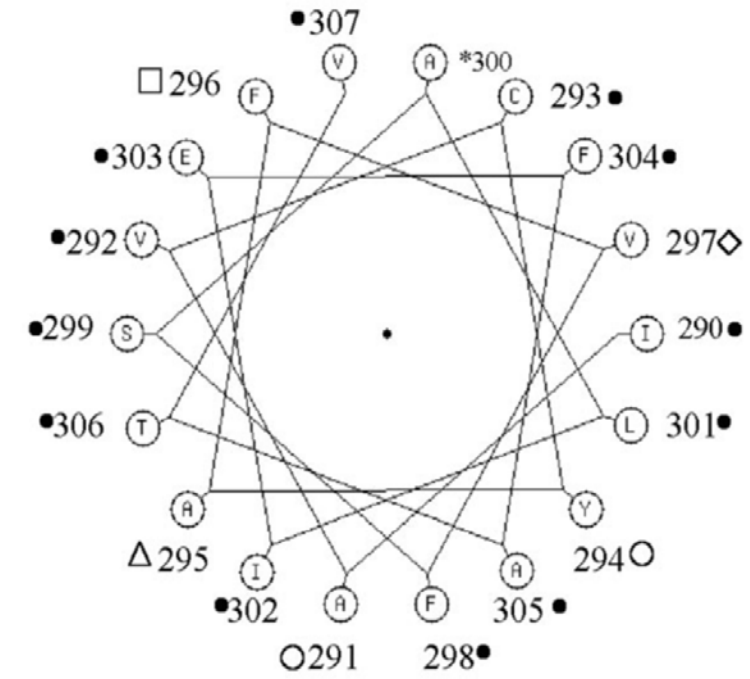
□, reactive with MTS in the alcohol- and GABA-bound states

◇ , reactive with MTS in the GABA-bound states

● , no effect

Figure 10. α-Helical representation showing the change of GABA-induced currents before and after treatment of MTS reagents on each Cys substitution mutant of TM3 in the absence and presence of GABA or alcohols. *A*, helical net projection. The *top side* shows extracellular end, and *bottom side* shows the intracellular end. Each symbol in *A* shows reactivity with MTS in indicated functional states. *, A300C receptor is not included because of little current response to GABA. The arrows indicate the movement of water-filled cavity.

B. α-helical wheel projection



○, reactive with MTS in the resting-, alcohol- and GABA-bound states

△, reactive with MTS in the alcohol-bound states

□, reactive with MTS in the alcohol- and GABA-bound states

◇ , reactive with MTS in the GABA-bound states

Figure 10. α-Helical representation showing the change of GABA-induced currents before and after treatment of MTS reagents on each Cys substitution mutant of TM3 in the absence and presence of GABA or alcohols. *B*, α-helical wheel projection. Each symbol in *B* shows reactivity with MTS in indicated functional states. *, A300C receptor is not included because of little current response to GABA.

Introduction of cysteines deeper than V297 within TM3 did not reveal any effect of MTS reagents in the absence or presence of GABA, ethanol or hexanol in our studies. This suggests that MTS reagents are not accessible to these amino acids, although we cannot rule out the alternative possibility that the modification of the amino acids by these alkyl MTS derivatives is functionally silent. It should be noted that some sites (F298C, S299C, L301C, I302C or V307C in the different states) appeared to be reactive to MTS reagents, but statistical comparison of wild type and these mutants did not show significant effects (Figures 7 and 8). Taken together, this suggests that the extracellular side of TM3 might be more flexible or dynamic compared to the intracellular side in the presence of agonist or modulator (Table 3). Recently, it is shown that the protein packing is loose around the extracellular half of the $GABA_A$ receptor β1 subunit TM2 in the presence of GABA (Goren et al., 2004), consistent with this study that the extracellular side of TM3 might be more flexible. This region composed of A291C, Y294C, A295C, F296C and V297C of TM3 may be involved in structural movements that transduce the allosteric modulation of $GABA_A$ receptor.

Table 3. Summary of reactivity of wild type and Cys mutants to MTS reagents and pCMBS in TM3.

	reactivity of MTS reagents in the following bound states EC_{10} and EC_{50} used for test-pulse				reactivity of pCMBS in the following bound states [a] saturating GABA and EC_{50} used for test-pulse				
mutant	resting	ethanol	hexanol	GABA	resting-	GABA	diazepam	potentiating propofol	activating propofol
$\alpha_1\beta_2\gamma_{2s}$	-	-	-	-	-	-	-	-	-
α_1(I290C)$\beta_2\gamma_{2s}$	-	-	-	-	nt	nt	nt	nt	nt
α_1(A291C)$\beta_2\gamma_{2s}$	*	*	*	*	*	*	*	nt	nt
α_1(V292C)$\beta_2\gamma_{2s}$	-	-	-	-	-	-	-	-	-
α_1(Y294C)$\beta_2\gamma_{2s}$	*	*	*	*	*	*	*	*	*
α_1(A295C)$\beta_2\gamma_{2s}$	-	*	*	-	-	-	-	-	-
α_1(F296C)$\beta_2\gamma_{2s}$	-	*	*	*	-	*	*	-	-
α_1(V297C)$\beta_2\gamma_{2s}$	-	-	-	*	-	-	-	*	-
α_1(F298C)$\beta_2\gamma_{2s}$	-	-	-	-	-	*	*	-	-
α_1(S299C)$\beta_2\gamma_{2s}$	-	-	-	-	-	-	-	-	*
α_1(A300C)$\beta_2\gamma_{2s}$ [b]	nt	nt	nt	nt	-	*	-	-	-
α_1(L301C)$\beta_2\gamma_{2s}$	-	-	-	-	-	*	*	-	-
α_1(I302C)$\beta_2\gamma_{2s}$	-	-	-	-	-	-	-	*	*
α_1(E303C)$\beta_2\gamma_{2s}$	-	-	-	-	-	*	-	-	*
α_1(F304C)$\beta_2\gamma_{2s}$	-	-	-	-	-	-	-	*	-
α_1(A305C)$\beta_2\gamma_{2s}$	-	-	-	-	-	-	-	-	*
α_1(T306C)$\beta_2\gamma_{2s}$	-	-	-	-	-	-	-	-	-
α_1(V307C)$\beta_2\gamma_{2s}$	-	-	-	-	-	-	-	-	-

- no effect, * reactive, nt: not tested, [a]data from Williams and Akabas (2002), [b]not tested due to little current response to GABA.

It is of interest to compare our data with those from previous studies which used the same mutants, but different thiol reagents (Williams and Akabas, 1999; Williams and Akabas, 2000). The mutants A291C, Y294C, F296C, F298C, A300C, L301C and E303C were found to be accessible to pCMBS in the presence of GABA. Our data showed that MTS reagents were reactive with A291C, Y294C, F296C and V297C in GABA-bound state. It should be noted that structurally and functionally different thiol reactive agents could show different accessibilities or reactivity with the same cysteine mutation or different reagents may have very different functional effects following modification (Pascual and Karlin, 1998; Reeves et al., 2001; Williams and Akabas, 1999; Zhang and Karlin, 1998). Furthermore, to investigate the accessibility GABA $EC_{5\text{-}10}$ or EC_{50} were used in this study and GABA EC_{50} or near-saturating GABA (Table 3) were used in the study from Williams and Akabas (Williams and Akabas, 1999), but the more important finding is that both studies show evidence for conformational changes in the TM3 of $GABA_A$ receptor $\alpha 1$ subunit induced by binding of agonist or modulators.

Chapter 4

Reaction rates of HMTS with accessible cysteine mutants in the resting, alcohol-bound or GABA-bound states

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Jung S, Akabas MH and Harris RA (2005) Functional and structural analysis of the $GABA_A$ receptor α1 subunit during channel gating and alcohol modulation. *J Biol Chem* 280(1):308-16.

4.1 Introduction

The initial accessibility experiments from chapter 3 have identified several amino acids positions that are water-accessible in the resting-, alcohol- or GABA-bound states in the TM3 of $GABA_A$ receptor $\alpha 1$ subunit. It is suggested that conformational changes by alcohol modulation or channel gating can transiently bring the cysteine into an aqueous environment and cause the cysteine to be ionized to be able to react with MTS reagents. The local environment of the introduced cysteine at these positions can be further characterized by investigating the rate constants of reaction of the accessible-cysteine mutants with MTS reagents. Additionally, the comparison of the rate constants in the different fuctional states can provide the structural insights on the microenvironment surrounding accessible cysteines in the specific functional state. The reaction rates of MTS reagents with the introduced cysteine sulfhydry group are mainly depending on collision frequency between MTS reagents and the ionized sulfhydryl group determined by (1) the access pathway from bulk solution to the cysteine and the local environment of sulfhydry group of the substituted cysteine residue, and (2) the sulfhydryl ionization state in the aqueous environment. Degree of the ionization state of the cysteine will be affected by how many water molecules contact to the substituted cysteine in the aqueous environment. It can be determined by the local concentration and residence time of the water molecules surrounding the introduced cysteine residue in the specific functional-state. (3) For the charged MTS reagents, the local concentration

and residence time of the reagents can be also dependent on the local electrostatic potential around the cysteine. The potential can also affect the ionization state of the introduced cysteine residue (Bera et al., 2002; Karlin and Akabas, 1998; Wagner and Czajkowski, 2001).

Neutral MTS reagent, HMTS was used in this chapter. Thus, it is likely that the reaction rates have been majorly determined by steric accessibility of the HMTS reagent to the sulfhydryl group and the sulfhydryl ionization state. If the introduced cysteine residue in the different functional-state become to be in a relatively open, aqueous environment, the cysteine residue will react faster than in a relatively restrictive, non-polar environment. To investigate the local environments surrounding reactive cysteines, reaction rates of the accessible mutants were determined.

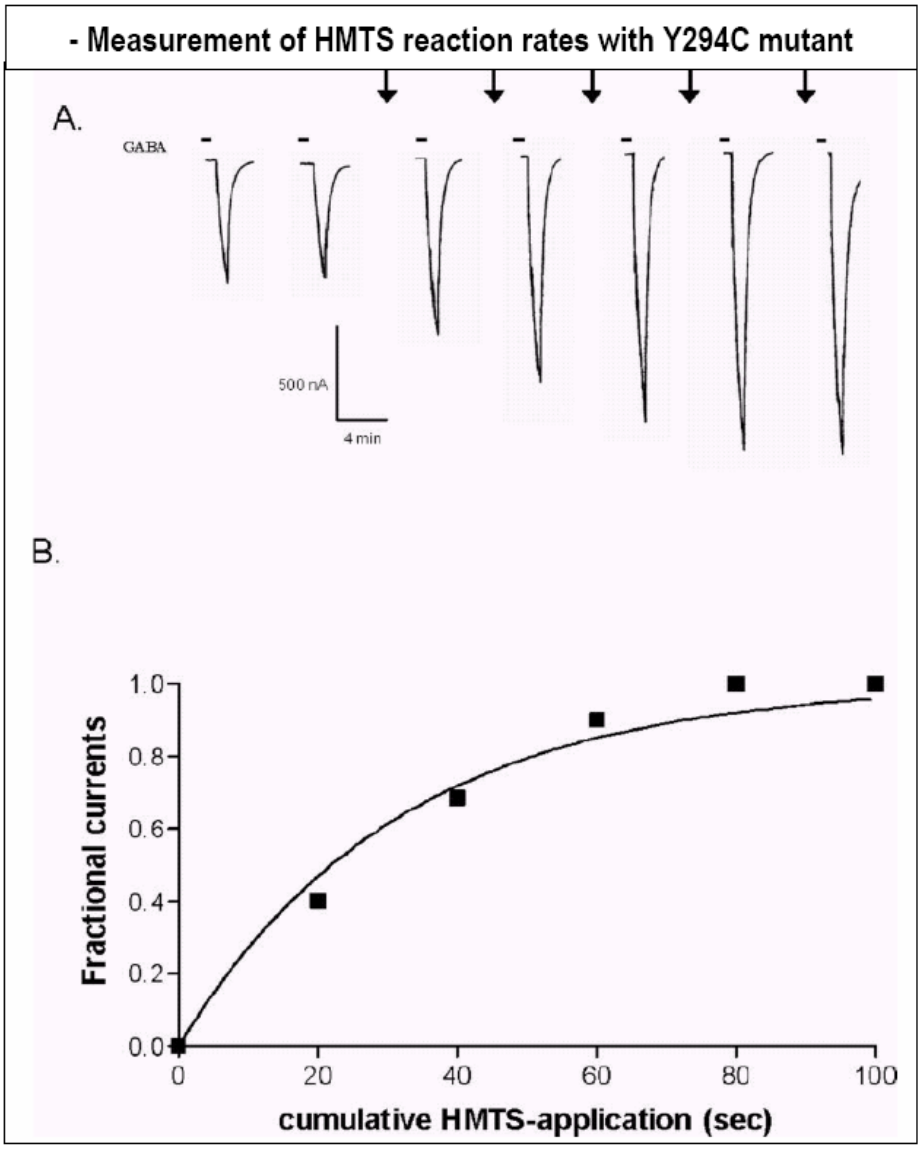

Figure 11. Measurement of MTS reaction rates with Y294C mutant. (A) Representative current tracing was obtained from an oocyte expressing Y294C mutant. $EC_{5\text{-}10}$ GABA-induced currents shown. Each arrow indicates application of 0.5 mM HMTS in the resting state for 20 sec. After 10 min washing, the GABA-induced current was redetermined. (B) Normalized currents to initial GABA-induced current are plotted against cumulative application time to HMTS and fitted with one phase exponential function. The calculated second order rate constants are shown in Table 3.

4.2 Reaction rates of HMTS with A291C and Y294C mutants in the resting, alcohol-bound or GABA-bound states

The A291C and Y294C mutants were accessible to HMTS in the resting state, suggesting that the water-filled cavity linked to extracellular solution exists surrounding A291 and Y294 is (Figure 8). To determine whether the physical environments around A291C or Y294C residues in the resting state are different from environments in the alcohol-bound or GABA-bound states, the rate of reactions of HMTS to A291C and Y294C mutants in the absence (Figure 11) and presence of alcohols or GABA were measured. Rate constants of A291C mutant were similar for resting, alcohol-bound and GABA-bound states (Table 4). This suggest that local environment around A291C in the resting state did not change significantly as the GABA- or alcohol-induced conformational changes undergo. Rate constants of Y294C mutant in the resting state showed similar ones in the alcohol-bound states, indicating that alcohol does not alter local conformation around Y294C residue during alochol modulation. However, Y294C mutant in the GABA-bound state reacted significantly faster with HMTS than in the resting or alcohol-bound states (Figure 12 and Table 4). This indicates that GABA-induced conformational changes around Y294C residue caused increased interaction between HMTS molecules and Y294C residue.

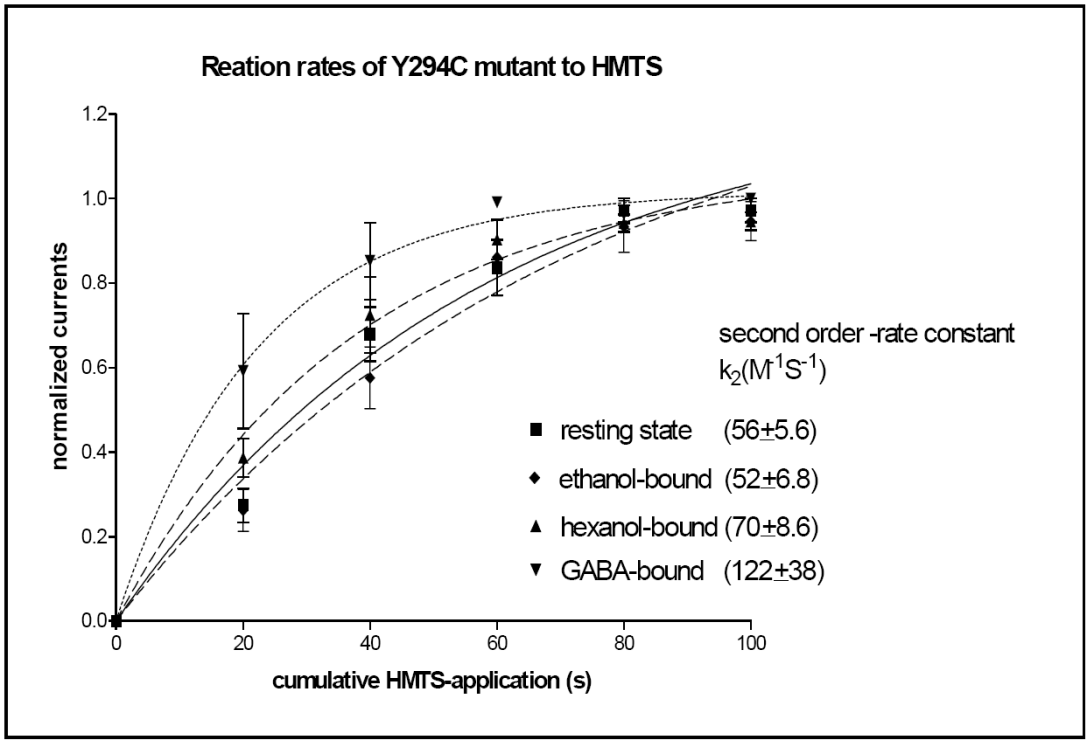

Figure 12. Reaction Rates of Y294C Mutant to HMTS in the resting (■), ethanol-bound (♦), hexanol-bound (▲), or GABA-bound (▼) states. Brief and sequential application of HMTS potentiated subsequent GABA-mediated (EC_{5-10}) currents. The normalized GABA-induced currents to the initial current were fitted to one phase exponential function and plotted as a function of cumulative HMTS exposure. Data were fit to a single exponential function to obtain a pseudo-first order rate constant (*k*). Second order rate constants (*k*2) were calculated by dividing the pseudo-first order rate constant by the concentration of HMTS used (0.5 mM). Data values represent the mean ± S.E. for at least 4 independent experiments.

Table 4. Second order rate constants of reactions of HMTS with accessib cysteine mutants in the resting, alcohol-bound and GABA-bound states.

mutants	resting -	GABA (1mM) -bound	hexanol (0.5mM) -bound	ethanol (200mM) -bound
A291C	69±8.0	60±4.4	74±3.0	66±5.7
Y294C	56±5.6	*122±38	70±8.6	52±6.8
A295C	–	–	71±25	98±25
F296C	–	62±9.0	50±15.6	82±15.4
V297C	–	50±3.6	–	–

– no reaction, * significant effect of GABA,
values are presented as mean±SEM from 4 to 7 oocytes.

4.3 Reaction rates of HMTS with A295C mutants in the alcohol-bound states

The A295C mutant did not react with HMTS in the resting state, whereas was accessible to HMTS only in the ethanol- and hexanol bound states (Figure 8). The results suggest that alcohol-induced conformational changes produce the A295C residue to be reactive with HMTS. Furthermore, the chapter 3 suggest that the water-filled cavity around A291 and Y294C might expand to this A295C residue by alcohol-induced conformational changes. The reaction rates of HMTS to A295C site were measured in the alcohol-bound states to investigate local environments of A295C in the alcohol-bound states. The second order rate constants of A295C mutant in the ethanol- and hexanol-bound states show 71$\pm$25 ($M^{-1}S^{-1}$) and 98$\pm$25 ($M^{-1}S^{-1}$), respectively (Table 4). These rate constants are not significantly different from each other, implying that microenvironment of around A295 in the ethanol-bound state is similar to one in the presence of hexanol. Additionally, the A295C mutant shows specific state-dependent accessibility of HMTS reagent in the presence of alcohols (Table 4).

4.4 Reaction rates of HMTS with F296C mutant in the alcohol-bound or GABA-bound states

The F296C mutant did not significantly react with HMTS in the resting sate,but became reactive to HMTS in the alcohol- and GABA-bound states (Figure 8). The F296C mutant showed specific state-dependent accessibility of MTS reagents in the presence of alcohols and GABA, respectively This suggests that alcohol- or GABA-induced conformational changes increase the reactivity of HMTS reagent to F296C residue. The reaction rates of HMTS to F296C site were measured in the alcohol- and GABA-bound states to compare local environments of F296C in the alcohol-bound states with one of GABA-bound state. The second rate constant of F296C mutant in presence of GABA was similar to rates in the presence of ethanol or hexanol (Table 4), indicating microenvironment of around F296 in the GABA-bound state is similar to ones in the presence of ethanol or hexanol (Table 4).

4.5 Reaction rates of HMTS with V297C mutant in the GABA-bound state

HMTS reagent was not accessible to the V297C mutant in the resting state and became accessible to the V297C mutant in the only GABA-bound state, implying that GABA-induced structural movements cause V297C residue to be reactive with HMTS (Figure 8). The V297C mutant showed specific state-dependent accessibility of MTS reagent in the presence of GABA. The second rate constant of V297C mutant shows 50 ± 3.6 ($M^{-1}S^{-1}$) (Table 4).

4.6 Discussion

4.6.1 Reaction rates of HMTS with the reactive mutants in the different fuctional states

The access pathway to the introduced cysteines and the sulfhydryl ionization state can be characterized by determining the rate constants of reaction with MTS reagents (Karlin and Akabas, 1998). The rate at which MTS reagents react with a cysteine side chain depends on the collision frequency between the MTS reagent and the ionized sulfhydryl group. The collision frequency depends on the local concentration of the MTS reagent in the vicinity of the cysteine. This is influenced by steric factors in the access pathway from bulk solution to the site of the cysteine. The extent to which a cysteine ionizes depends on fractional time that the residue is in contact with water, bulk solution pH or on the local electrostatic potential. It is very difficult to distinguish which of the above factors affects the accessiblity in the different fuctional states, and has been suggested that the reaction rates are mainly determined by steric accessibility pathway of the MTS reagents to the sulfhydryl group and the sulfhydryl ionization state (Karlin and Akabas, 1998; Wagner and Czajkowski, 2001).

To investigate the local physical environment of the accessible cysteines introduced in the TM3 of the $GABA_A$ receptor $\alpha 1$ subunit, the rate constant of reaction of HMTS to each of the reactive cysteine-mutants was determined. The rate

constants of the A291C mutant did not vary in the different functional-states with treatments of HMTS, suggesting that the local environment around the A291C residue in the alcohol-bound states did not change significantly from environment in the resting- or GABA-bound states. The A291C residue is accessible to HMTS in the resting state, indicating that this position is connected to extracellular solution (chapter 3), and the local environment surrounding A291C is relatively open and aqueous. The Y294C mutant is also reactive to HMTS in the resting state, suggesting that the water-filled cavity around this site is linked to one of A291C. The rate constant of the Y294C mutant in the GABA-bound state is significantly faster than the rate constants in the resting or alcohol-bound states. This indicates that the local structure around Y294C and/or the access pathway stabilized by GABA is different from the resting and alcohol-bound states. This is consistent with previous results with the Y294C mutant (Williams and Akabas, 2000). Thus, this indicates that GABA-induced rotation and/or tilting of α1 subunit make local environment surrounding Y294 position relatively more open and/or aqueous, consistent with that the structural volume of water-filled cavity in the protein interior changes as proteins undergo conformational changes (Gottschalk et al., 2001; Whittington et al., 2001). The increased rate constant by GABA binding indicates that this region undergoes a conformational change during channel gating.

F296C mutant is accessible to MTS reagents in the alcohol- and GABA-bound states. The reaction rate of the F296C mutant with HMTS in the GABA-

bound state is not significantly different from reaction rate in the alcohol-bound states. Therefore, the microenvironment surrounding the F296C residue induced by alcohol-binding seems to be similar to local environment induced by GABA-binding. The rate constant of reaction of the F296C mutant in the GABA-bound state is significantly smaller than one of Y294C in the presence of GABA. This suggests that enlarged water-filled cavity around Y294 residue become smaller one at F296 site during GABA-induced conformational changes. The V297C mutant is accessible to HMTS only in the GABA-bound state, indicating that only GABA-induced conformational changes cause this position to be reactive with HMTS. The rate constant of reaction of the V297C mutant in the GABA-bound state is similar to one of F296C in the presence of GABA, indicating that the size of the water-filled cavity would not be changed as the cavity expands to V297 residue during channel gating.

Chapter 5

S270 and A291 Sites in TM2 and 3 are Critical for Alcohol-Induced Conformational Changes in $GABA_A$ Receptor α_1 Subunits

5.1 Introduction

Substituted cysteine accessibility method (SCAM) has been used to investigate the structure and conformational changes of the transmembrane (TM) region of $GABA_A$ receptor α1 subunit during channel gating and actions of modulators (Boileau et al., 2002; Jung et al., 2005; Karlin and Akabas, 1998; Lobo et al., 2004a; Lynch et al., 2001; Williams and Akabas, 1999; Williams and Akabas, 2000; Williams and Akabas, 2002). Our preliminary results showed that the A295C and F296C mutants in TM 3 of the $GABA_A$ receptor α1 subunit were not reactive to MTS reagents in the resting state, but accessible to MTS reagents in the presence of alcohols (Jung et al., 2005), suggesting that conformational changes induced by alcohol cause the water-accessible surface of the TM3 segment to increase from the region around A291C and Y294C to the deeper region surrounding A295C and F296C. Furthermore, the study indicated that the extracellular side of TM3 is flexible or dynamic during alcohol-modulation. It has been shown that a binding cavity for alcohols may exist in a crevice near the extracellular ends of the TM2 or TM3

regions of $GABA_A$ receptor, and S270 and A291 residues are particularly critical sites of alcohol-binding pocket (Mascia et al., 2000b; Mihic et al., 1997; Wick et al., 1998). However, it is unknown how alcohol-induced conformational movements between TM regions or within TM domain during alcohol modulation are regulated. In this study, we investigated how the mutations of S270 or A291 affect alcohol-induced conformational changes by testing the accessibility of HMTS to accessible A295C or F296C mutants in the alcohol-bound states.

Previous results have shown that point mutations of S270I or A291W in the TM2 or TM3 regions of $GABA_A$ receptor α subunit drastically abolished ethanol-induced potentiations of the GABA-induced responses (Mihic et al., 1997; Ueno et al., 2000; Ueno et al., 1999b). In this study, to investigate the effect of S270I or A291W mutations on the accessibility of A295C or F296C TM3 mutants to HMTS reagent in the alcohol-bound states, the accessibilities of the double mutants (S270I/A295C, S270I/F296C, A291W/A295C or A291W/F296C) to HMTS reagent in the presence of ethanol or hexanol were investigated using SCAM (GABA-induced currents in oocytes expressing the double mutants (S270I/A295C, S270I/F296C, A291W/A295C or A291W/F296C) $GABA_A$ receptors were determined before and after treatment of MTS reagents with ethanol or hexanol to test if mutations (S270I or A291W) of critical amino acids of alcohol-binding pocket affect the accessibility of MTS reagents to A295C and F296C mutants in the alcohol-bound states). There were significant reduced accessibilities of HMTS reagent to all

the double mutants in the ethanol-bound state, and to S270I/F296C, A291W/A295C or A291W/F296C double mutants in the hexanol-bound state. Therefore, these results support the finding that amino acids S270 and A291 in the $GABA_A$ receptor $\alpha 1$ subunit provide the sites responsible for the alcohol-binding pocket.

5.2 Expression and functional characterization of double mutants

The $\alpha_1\beta_2\gamma_{2S}$-combination of subunits for double mutant $GABA_A$ receptors expressed in *Xenopus* oocytes in this study was used as described in chapters 3 and 4. To assess whether double mutations affected $GABA_A$ receptor function, each mutant $\alpha 1$ subunit was coexpressed with wild-type β_2 and γ_{2S} subunits in oocytes. Concentration-response relationships of GABA were determined from the mutants and all of the double mutants produced functional GABA-induced currents (Figure 13 and Table 5), indicating that all of the mutant subunits assembled into the receptors and the double mutations were well tolerated. The three double mutants (S270I/A295C, A291W/A295C and A291W/F296C) showed increased GABA sensitivity (5- to 10-fold shifts) relative to wild type

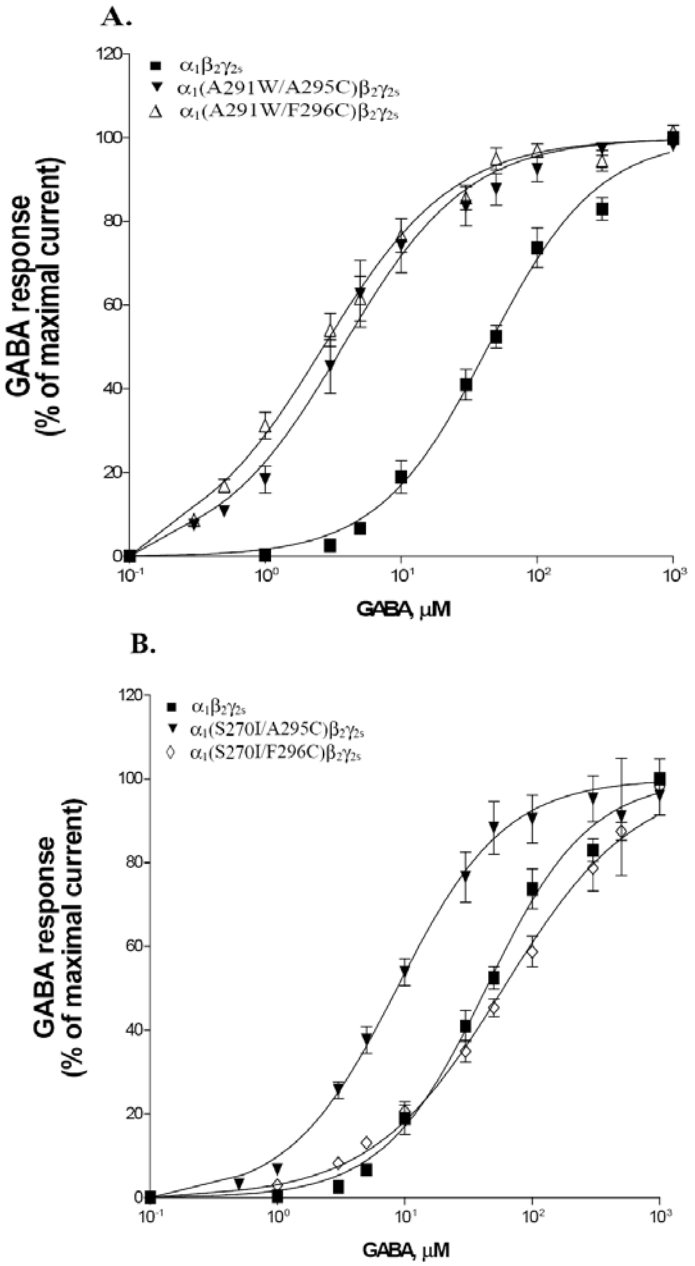

Figure 13. GABA concentration-response curves for wild-type and mutant $GABA_A$ receptors expressed in *Xenopus* oocytes. The curves for mutant α_1(A291W/A295C)$\beta_2\gamma_{2s}$ and α_1(A291W/F296C)$\beta_2\gamma_{2s}$ are shown in (A) and mutant α_1(S270I/A295C)$\beta_2\gamma_{2s}$ and α_1(S270I/F296C)$\beta_2\gamma_{2s}$ are in (B). The curve for wild-type $\alpha_1\beta_2\gamma_{2s}$ is shown in both (A) and (B). Nonlinear regression analysis of the curves was performed as described in Methods and Materials, and the results are summarized in Table 5. Data represent the mean $\pm$ S.E. from four to seven oocytes.

Table 5. GABA EC_{50}, Hill coefficients and maximum currents for the wild type and mutant $GABA_A$ receptors

mutant	EC_{50} (μM)	Hillslope	I_{max} (nA)
$\alpha_1\beta_2\gamma_{2s}$	**44.9±4.58**	**1.34±0.07**	**3954±435**
α_1(**S**270I)$\beta_2\gamma_{2s}$	**6.3±0.82**	**0.92±0.13**	**1126±178**
α_1(**A**291W)$\beta_2\gamma_{2s}$	**0.44±0.09**	**1.2±0.10**	**1090±162**
α_1(**A295C**)$\beta_2\gamma_{2s}$	**123±10**	**1.50±0.17**	**1406±160**
α_1(**F296C**)$\beta_2\gamma_{2s}$	**111±10**	**1.26±0.11**	**1180±327**
α_1(**S270I/A295C**)$\beta_2\gamma_{2s}$	**8.2±2.75**	**1.18±0.26**	**3133±422**
α_1(**S270I/F296C**)$\beta_2\gamma_{2s}$	**59±9.1**	**0.82±0.1**	**662±81**
α_1(**A291W/A295C**)$\beta_2\gamma_{2s}$	**3.9±0.84**	**1.3±0.13**	**1867±397**
α_1(**A291W/F296C**)$\beta_2\gamma_{2s}$	**2.9±0.50**	**1.1±0.06**	**1650±165**

Values are presented as mean±S.E. from 4 to 8 oocytes.

and a double mutant (S270I/F296C) showed similar GABA sensitivity to wild type receptor (Table 5). Next, the effects of ethanol and hexanol on the double mutants were determined. The potentiating effects of 200mM ethanol on GABA EC_{5-10}-induced currents were abolished in all the S270I- and A291W-containing double mutants (Table 6). For all the double mutants, the potentiating effect of 0.5mM hexanol on GABA EC_{5-10}-induced currents were also abolished or significantly reduced compared to wild-type receptors (Table 6). These alcohol concentrations (200mM ethanol or 0.5mM hexanol) approximately correspond to the anesthetic concentration *in vivo* (Alifimoff et al., 1989; Fang et al., 1997)

Table 6. Potentiation of currents induced by GABA (EC_{5-10}) by ethanol or hexanol for wild type and mutant $GABA_A$ receptors.

mutant	% potentiation ethanol (200mM)	hexanol (0.5mM)
$\alpha_1\beta_2\gamma_{2s}$	67±5.3	70±2.9
α_1(S270I)$\beta_2\gamma_{2s}$	1.1±3.0	32.4±4.6
α_1(A291W)$\beta_2\gamma_{2s}$	1.1±2.2	5.2±2.4
α_1(A295C)$\beta_2\gamma_{2s}$	20±3.8	75±14.4
α_1(F296C)$\beta_2\gamma_{2s}$	20±1.1	55±4.5
α_1(S270I/A295C)$\beta_2\gamma_{2s}$	1.6±1.8	44.8±5.3
α_1(S270I/F296C)$\beta_2\gamma_{2s}$	-7.8±2.5	22±2.5
α_1(A291W/A295C)$\beta_2\gamma_{2s}$	12±2.9	17±2.4
α_1(A291W/F296C)$\beta_2\gamma_{2s}$	3±1.8	17±2.8

Values are presented as mean±S.E. from 4 to 8 oocytes.

5.3 Effects of A291W on the accessibility of HMTS to A295C and F296C mutants

The A295C and F296C sites became reactive to HMTS reagent in the alcohol-bound states by alcohol-induced conformational changes, but not in the resting state (Jung et al., 2005). It has been shown that S270I or A291W point mutations abolished ethanol potentiation, suggesting these sites are essential residues for alcohol action, and furthermore, S270 or A291 residues are critical sites for alcohol-binding pocket (Mascia et al., 2000b; Mihic et al., 1997; Wick et al., 1998). To assess whether mutations of the critical amino acids of alcohol-binding pocket affect alcohol-induced conformational changes, the accessibility of HMTS reagent to the A291W/A295C and A291W/F296C or the S270I/A295C and S270I/F296C mutants were investigated in the alcohol-bound states.

First, to investigate whether the A291W mutation of TM3 affects alcohol-induced conformational changes, the accessibility of HMTS to the A291W/A295C and A291W/F296C double mutants were determined by examining changes of GABA-induced currents of the double mutant receptors after application of HMTS in the presence of 200mM ethanol or 0.5mM hexanol. The A291W/A295C and A291W/F296C double mutants showed abolished ethanol potentiation and markedly reduced hexanol potentiation on $EC_{5\text{-}10}$ GABA-induced currents (Table 6).

Previous data showed that the GABA EC_{5-10}-induced currents of the A295C and F296C mutants were significantly changed after application of HMTS reagent with 200 mM ethanol or 0.5 mM hexanol, suggesting that alcohol-induced conformational changes caused A295C or F296C sites to be accessible to HMTS (Jung et al., 2005). First, A291W/A295C and A291W/F296C double mutants did not react with the HMTS reagent in the resting state (Figures 14 and 15). Furthermore, the A291W/A295C and A291W/F296C double mutants had no significant effects of HMTS on the GABA-induced currents in the ethanol- and hexanol-bound states (Figures 14 and 15). The results suggest that the A291W mutation of TM3 affects alcohol binding to reduce alcohol-induced conformational changes, resulting in significantly reduced the accessibility of HMTS to A295C and F296C sites in the TM3 region in the ethanol- and hexanol-bound states.

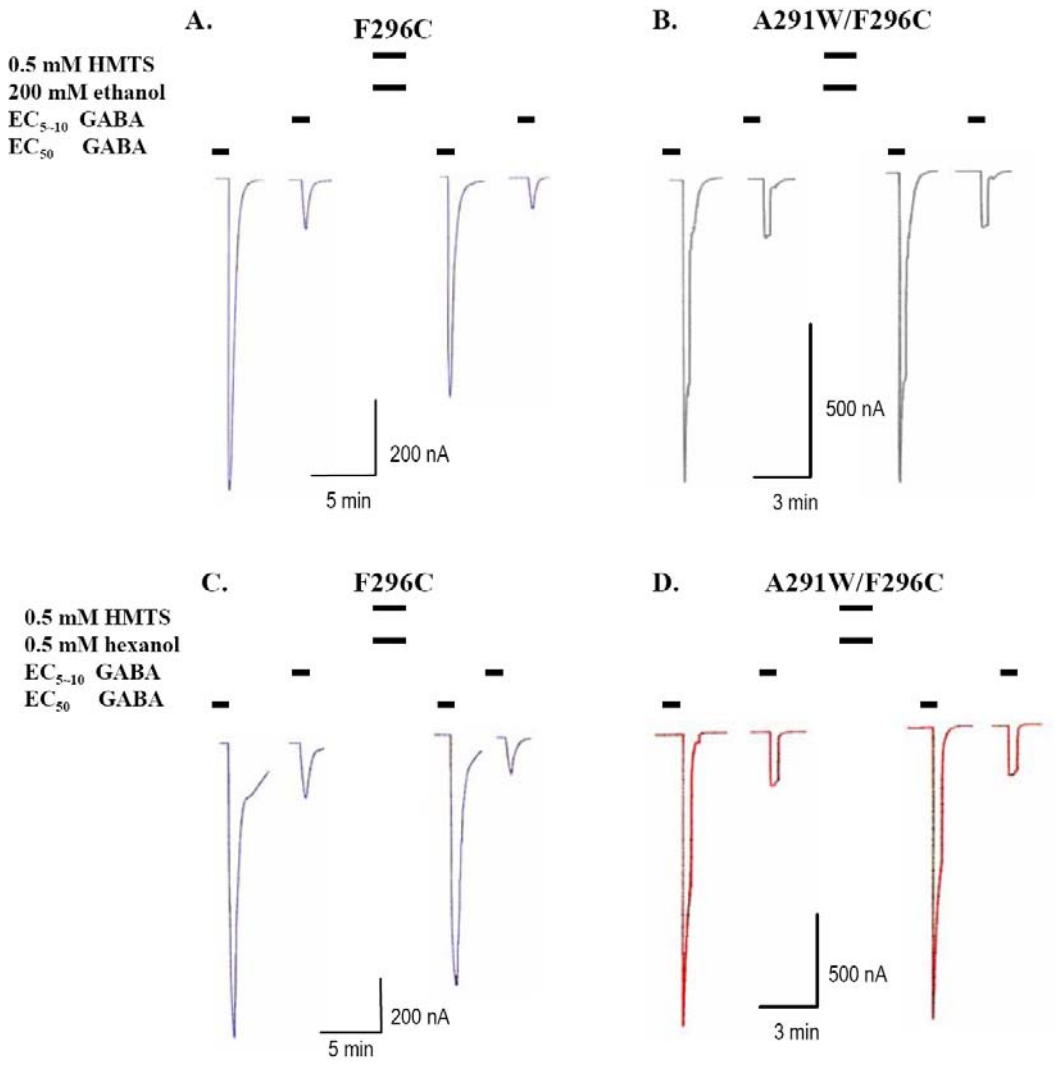

Figure 14. A291W mutation reduced the accessibility of HMTS to F296C site in the alcohol-bound states. Representative tracings obtained from oocytes expressing (A) F296C and (B) A291W/F296C mutants in the ethanol-bound states, (C) F296C and (D) A291W/F296C mutants in the hexanol-bound sates, suggesting that the mutation of critical site (A291) of alcohol-binding pocket abolishes alcohol-induced conformational changes to reduce reactivity of HMTS to F296C residue, which was accessible to HMTS in the alcohol-bound states. (A) inhibitory effect of HMTS applied in the ethanol-bound state, (B) no effect of HMTS applied in the ethanol-bound state, (C) inhibitory effect of HMTS applied in the hexanol-bound state, and (D) no effect of HMTS applied in the hexanol-bound state.

5.4 Effects of S270I on the accessibility of HMTS to A295C and F296C mutants

The S270I/A295C and S270I/F296C double mutants were not accessible to HMTS in the resting state (Figure 15). Then, co-application of HMTS with 200mM ethanol had no significant effects on the S270I/A295C and S270I/F296C double mutants (Figure 15). Additionally, GABA-induced currents of the S270I/F296C double-mutant receptors were not significantly changed after applying HMTS reagents with 0.5mM hexanol (Figure 15). These results might reflect that potentiating effects of 200mM ethanol were markedly reduced or abolished in the S270I/A295C and S270I/F296C double mutants, and effect of 0.5mM hexanol on the S270I/F296C mutant was also dramatically reduced (Table 6). Thus, these results suggest that the S270I mutation affects alcohol binding to prevent these double mutants from being reactive with HMTS reagent by alcohol-induced conformational changes.

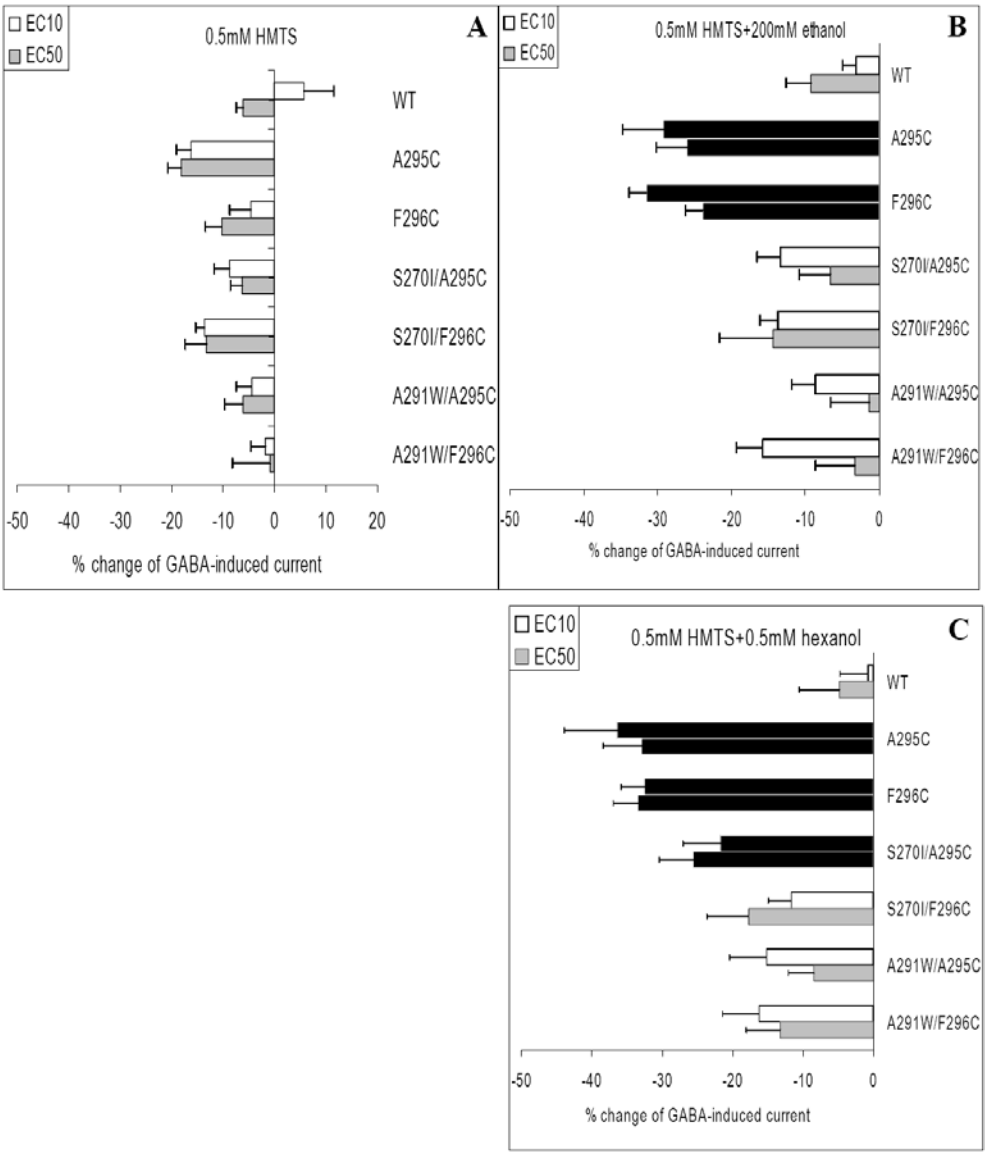

Figure 15. Effects of HMTS on GABA-induced currents of wild-type and mutants $GABA_A$ receptors in the resting state (A), ethanol-bound state (B), or hexanol-bound state (C). For GABA-induced currents, the GABA EC5~10 (top bar, white) and EC50 (lower bar, gray) were applied on wild-type and mutant GABAA receptors. Black bars indicate effects that are significantly different statistically from the effect on wild type in each condition by a one-way ANOVA, using the Dunnet post hoc test. The % change was calculated as = $\{I_{after}/I_{before}) - 1\} \times 100$, where I_{before} and I_{after} indicate the values of the two GABA-induced currents before and after the application of the sulfhydryl reagent (0.5 mM applied for 90 sec). All values are presented as mean $\pm$ S.E. from 3 to 8 oocytes.

However, treatment with HMTS in the presence of hexanol significantly inhibited GABA-induced currents at the S270I/A295C double mutant (Figure 15), indicating that the S270I/A295C mutant was reactive to HMTS in the presence of 0.5mM hexanol. The S270I/A295C mutant significantly reduced hexanol potentiation, but still showed 45% hexanol potentiation (compared to 70% potentiation for wild-type) (Table 6). Thus, this degree of hexanol potentiation may be sufficient to allow the S270I/A295C mutant to be accessible to HMTS in the presence of hexanol. Taken together, these results indicate that the S270I mutation of TM2 region significantly reduced the accessibility of HMTS to A295C and F296C residues in the TM3 region in the ethanol-bound state, and to F296C residue of TM3 domain in the hexanol-bound state (Figure 15).

5.5 Discussion

Previous results suggested that both the Ser270 and Ala291 in the TM2 and TM3 regions of $GABA_A$ receptor α subunit are essential amino acids for actions of alcohols and volatile anesthetics, and mutations such as S270I or A291W completely eliminate ethanol-induced potentiation on the GABA-induced currents (Mihic et al., 1997). Furthermore, the existence of alcohol-binding pocket in the GABA and glycine receptors has been inferred from studies of the cutoff in activity in the *N*-alkanols as alcohol-chain length increases, and the S270 and A291 residues (equivalent sites, S267C and A288C residues in the homologous glycine receptor) are shown to be critical amino acids of the alcohol-binding pocket (Dildy-Mayfield et al., 1996; Mascia et al., 2000b; Wick et al., 1998). Recently, equivalent sites, S267C and A288C residues in the homologous glycine receptor are shown to be close and facing each other (Lobo et al., 2004c). Additionally, our results suggested that conformational changes induced by ethanol or hexanol in the TM3 domain of $GABA_A$ receptor α1 subunit cause a water-filled cavity surrounding A291 and Y294 sites located in extracellular end to expand into lower A295 or F296 residues, and this expansion of the water-filled cavity made the A295C or F296C residues to be reactive MTS reagents in the alcohol-bound states (Jung et al., 2005).

5.5.1 Effects of S270I or A291W mutations on alcohol potentiation

Previous studies showed that in the GABA and homologous receptors, point mutations of S270 or A291 residues (equivalent residues of homologous receptors) in the TM2 or TM3 domains to larger amino acids result in decreased the alcohol cutoff. These data suggest that the molecular volumes of the amino acid residues, S270 or A291 (equivalent residues, S267 or A288 of glycine receptor α_1 subunit) of alcohol-binding pocket are negatively correlated with the potentiation produced by alcohols and the amino acid residues can control the size of the alcohol-binding pocket. (Koltchine et al., 1999; Wick et al., 1998; Ye et al., 1998). In this study, all double mutants (A291W/A295C, A291W/F296C, S270I/A295C and S270I/F296C) showed abolished potentiation of GABA-induced currents by 200mM ethanol. In addition, the potentiating effects of 0.5mM hexanol on those double mutants significantly decreased (range of 17% - 44%, compared 70% for wild type), but significant hexanol potentiation remained for S270I, S270I/A295C and S270I/F296C mutants.

It is of interest to note that the cutoff for the actions of *n*-alkanols on the $GABA_A$ receptor and related receptors (glycine and GABA α1 receptors) occur between decanol (molecular volume of 234 $Å^3$) and dodecanol (molecular volume of 276 $Å^3$) (Dildy-Mayfield et al., 1996; Wick et al., 1998), providing an estimated molecular volume of alcohol-binding pocket of about 270 $Å^3$. Molecular volumes of

ethanol and hexanol are 70 and 152 $Å^3$ (Ueno et al., 1999a). For the S270I mutation, the difference in volume of the serine and isoleucine residues indicates that the pocket is partially filled by about 53 $Å^3$, assuming that no gross distortion of secondary structure occurs (Wick et al., 1998). Consequently, molecular size of the pocket can be thought to decrease to about 217 $Å^3$ (= 270-53 $Å^3$) but it can still accommodate hexanol (molecular volume of 152 $Å^3$). For the A291W mutant, all potentiation of the GABA-induced responses by ethanol and hexanol were abolished. This suggests that the large molecular volume of tryptophan might occupy most of the alcohol-binding pocket to prevent ethanol or hexanol from binding to the pocket. A291W will bring an addition of 125 $Å^3$ (Wick et al., 1998) to the pocket to reduce molecular volume of the pocket to about 145 $Å^3$ (= 270-125 $Å^3$). Therefore, this large decrease in the size of the cavity may not allow hexanol (molecular volume of 152 $Å^3$) to fit into the cavity. This would explain why single or double mutants containing S270I mutation showed remaining hexanol potentiation, but potentiations of GABA-induced responses by hexanol were abolished at single or double mutants containing A291W mutation.

Effects of ethanol on GABA responses are more sensitive to mutation than those of hexanol and not clearly explained by the volume changes. Ethanol-binding to the pocket might require at least correctly oriented both Ser270 and Ala291 residues of alcohol-binding pocket, and additionally specific hydrogen-bonding involved in Ser270 of TM2, consistent with that A291C mutant showed abolished

ethanol potentiation although there is still enough room for ethanol-binding (Jung et al., 2005), and S267C or A288C mutants in equivalent positions of glycine receptor also showed abolished ethanol potentiation, but produced similar octanol potentiation to wild type glycine receptor (Lobo et al., 2004c; Mascia et al., 2000b). In addition, previous structural data of LUSH protein (alcohol-binding protein of *Drosophila*) suggested that binding of short-chain alcohols such as ethanol may require alcohol-binding cavity containing correctly oriented hydrogen-bonding groups, whereas nonpolar interactions that would be involved in smaller hydrogen-bonding interactions in the cavity may mainly play a important role in binding of long-chain alcohols to obtain the same overall binding affinity as ethanol (Kruse et al., 2003). It should be noted that hexanol has much greater hydrophobicity than ethanol molecule.

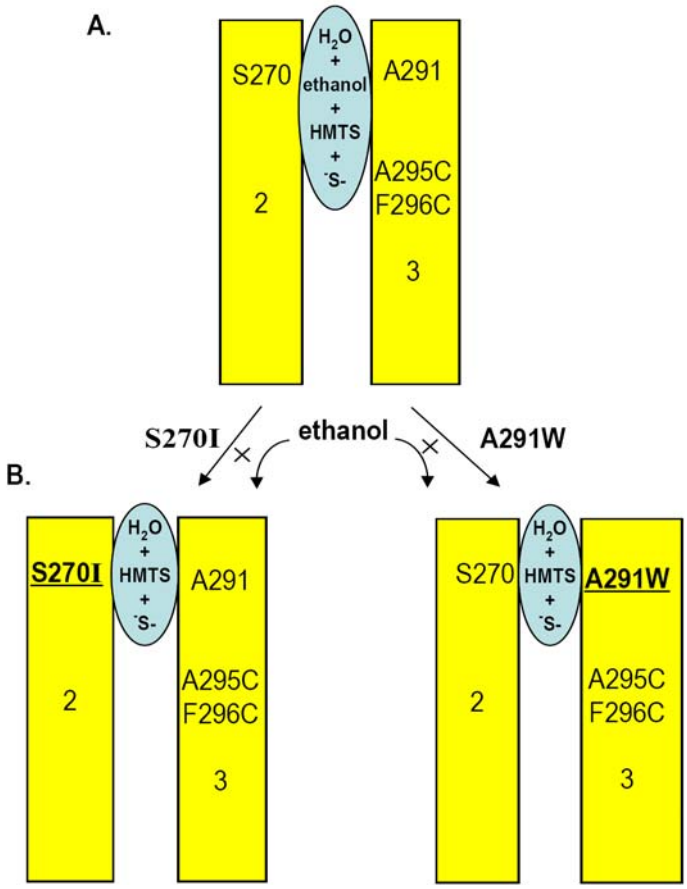

Figure 16. A schematic illustrating the effects of S270I or A291W mutations on accessibility of HMTS to A295C or F295C sites (A) Ethanol-binding to the intact alcohol-binding pocket induced the water-filled cavity to expand to A295C or F296C sites to be reactive with HMTS reagent. (B) S270I or A291W mutations abolished ethanol binding to the pocket, resulting in no expansion of the cavity toward A295C or F296C residues. Putative membrane-spanning helices are shown as cylinders and only TM2 and 3 are shown for clarity.

5.5.2 Effects of S270I or A291W mutations on accessibility of HMTS to A295C or F296C sites

In the A291W/A295C, A291C/F296C, S270I/A295C, or S270I/F296C double mutants, the S270I or A291W mutations significantly reduced the accessibility of HMTS to A295C or F296C sites in the presence of ethanol, indicating that the S270 or A291 residues as critical sites for ethanol action. These results suggest that S270I or A291W mutations of alcohol-binding pocket affect ethanol binding to $GABA_A$ receptor $\alpha 1$ subunit to reduce ethanol-induced conformational changes, which lead to prevent the water-filled cavity lined by A291/Y294 containing HMTS applied from expanding to lower A295C or F296C sites (Figure 16). Furthermore, the accessibility of HMTS to the A291W/A295C, A291C/F296C or S270I/F296C double mutants significantly decreased in the hexanol-bound state, but the S270I/A295C mutant is still reactive to HMTS with hexanol. It should be noted that the S270I/A295C mutant still showed potentiation (45%) by hexanol. This degree of potentiation might be enough to induce conformational change by hexanol to cause the mutant to be reactive to HMTS in the hexanol-bound state. The A291W/A295C, A291C/F296C and S270I/F296C double mutants showed significantly reduced potentiations (17% - 22%) by 0.5mM hexanol. These double mutants were not reactive to HMTS in the presence of hexanol. It is unlikely that these degrees of potentiation by hexanol might induce conformational

changes in the $GABA_A$ receptor to increase the accessibility of HMTS to the introduced cysteines. In the present study, application of 0.5mM hexanol to the S290I/A295C mutant showed higher potentiation than to the S270I/F296C mutant. It is very difficult to interpret structurally what additional effects the A295C or F296C mutations provide on the S270I mutation for the hexanol action in the S270I/A295C or S270I/F296C double mutants, but one possible interpretation of the results would be that the F296C mutation in the S270I/F296C mutant might produce an additional effect on binding of hexanol and thus, result in lower potentiation by hexanol than the S270I/A295C mutant.

In summary, these data support previous findings that Ser270 and Ala291 are essential amino acids in alcohol-binding pocket lined by at least TM2 and TM3 domains (possibly TM1 or TM4 domains together) for actions of alcohols in the $GABA_A$ receptor $\alpha 1$ subunit. Additionally, these results imply that the S270 or A291 residues would affect alcohol-induced conformational movements between TM2 and TM3 or within TM3 domain during alcohol modulation in the $GABA_A$ receptor $\alpha 1$ subunit. Furthermore, the A291 or S270 amino acid residues of alcohol-binding pocket in the TM2 and TM3 of $GABA_A$ receptor $\alpha 1$ subunit may have an important role in both forming the binding sites for alcohols and in the mechanism of allosteric modulation of the $GABA_A$ receptor by alcohols.

Chapter 6

Discussion

Jung S, Akabas MH and Harris RA (2005) Functional and structural analysis of the $GABA_A$ receptor α1 subunit during channel gating and alcohol modulation. *J Biol Chem* 280(1):308-16.

6.1 The water-filled cavity in the $GABA_A$ receptor α1 subunit

The results presented in Chapter 3 and 4 suggest that water-filled cavity, which is located in upper region of TM3 in the $GABA_A$ α1 subunit, expanded to lower part of TM3 during channel gating and alcohol modulation. First, the A291C and Y294C mutants are accessible to MTS reagents in all functional states including resting state. The results indicate that these positions are water-accessible, and this water-filled cavity lined partly by A291 and Y294 residues is believed to be directly connected to extracellular solution because water-soluble MTS reagents are applied extracellularly on oocytes expressing the mutant $GABA_A$ receptors. The A291 residue has been shown to be one of critical sites of binding pocket for alcohol and volatile anesthetics (Jenkins et al., 2001; Mihic et al., 1997; Ueno et al., 1999b).

The results indicate that the A295C and F296C sites are not accessible to MTS reagents in the resting state, but became water-accessible in the presence of ethanol or hexanol. The data suggest that alcohol-induced conformational changes cause the water-filled cavity around A291 and Y294 to expand to the A295C and F296C residues to be exposed to MTS reagents. Then, the covalent modifications of MTS reagents to the A295C and F296C positions alter the functions of the cysteine mutants to be detected electrophysiologically. Interestingly, the F296C mutant is also reactive MTS reagents in the GABA-bound state. This suggests that the F296 position might be involved in transducing conformational changes induced by both of GABA and alcohols. Furthermore, the V297C mutant does not react with MTS

reagents in the resting state, but became reactive with MTS reagents in the only presence of GABA. It has been suggested that the binding of agonist to extracellular N-terminal domain induced structural movements, and these conformational changes are transferred to transmembrane regions, resulting in rotation and tilting of TM domains, which produce channel gating (Kash et al., 2003; Miyazawa et al., 2003).

It has been suggested that a water-filled cavity is present in the resting state and the cavity is lined in part by the A291 and Y294 residues of TM3 region, and the S270 residue of TM2 region of α1 subunit (Mascia et al., 2000b; Williams and Akabas, 1999). It is unknown whether this cavity around the extracellular side of TM3 segment is continuously present because the proteins are fluctuating dynamically. Interestingly, it has shown that a cavity region in the interior of protein could accommodate up to several water molecules (Yu et al., 1999). Furthermore, the exchange of internal water molecules of a protein with bulk water has been suggested to be mediated by conformational fluctuations in the protein (Dencher et al., 2000; Gottschalk et al., 2001). It is interesting to note that crystal or spectroscopic structural analyses of some proteins complexed with anesthetics have identified pre-existing cavities in the proteins (Bhattacharya et al., 2000; Eckenhoff et al., 2001; Franks et al., 1998; Whittington et al., 2001).

However, the cavity at least transiently exists and is connected to extracellular solution because the A291C, Y294C and S270C mutants react with sulfhydryl-specific reagents in the resting state (Jung et al., 2005; Mascia et al.,

2000b; Williams and Akabas, 1999). In the presence of GABA or modulators, the cavity located in extracellular side of the TM3 domain extends deeper into lower part of the TM3 region.

Based on results of functional accessibility and reaction rate of MTS reagents in this study, our data also suggest that the extracellular side of TM3 is more flexible or dynamic during channel gating or alcohol-modulation and the cytosolic side of TM3 might adopt a more tightly packed or rigid conformation. Characterizing reaction rates to describe local microenvironments surrounding the accessible cysteines in the different functional-states suggest that the conformations produced by GABA or alcohols are similar but not identical.

The 4 Å resolution structure of the homologous *Torpedo* ACh receptor by cryoelectron microscopy provides presents a model of the closed pore. The ion channel pore is surrounded by an inner ring of 5 α-helices from the five TM2 regions of each of the five subunits and the inner ring forms the primary channel lining. An outer ring of α-helices formed by TM1, TM3 and TM4 is loosely packed against the inner ring (pore-lining helices, TM2). Furthermore, the model suggests that TM2 is mainly separated from TM1 or TM3 by water-filled space, potentially allowing water to enter the space (Miyazawa et al., 2003; Unwin, 2005).

The S270 and A291 residues in the TM2 and TM3 regions have been shown to be critical for alcohol and volatile anesthetic action and hypothesized to line a binding pocket for alcohols and volatile anesthetics between, at least, the TM2 and

TM3 segments in the $GABA_A$ receptor α subunit (Jenkins et al., 2001; Koltchine et al., 1999; Mascia et al., 2000b; Mihic et al., 1997; Ueno et al., 2000; Ueno et al., 1999b). The results (chapter 5) showed that all double mutants (A291W/A295C, A291W/F296C, S270I/295C, and S270I/F296C) abolished potentiations of the GABA-induced responses by ethanol and were not accessible to HMTS in the ethanol-bound state, suggesting that the S270 and A291 residues are important sites for alcohol-induced conformational changes. It can be explained that reduced binding of ethanol results in decreased ethanol-induced conformational changes, which prevent the water-filled cavity from expanding toward the A295C or F296C residues. Thus, the A295C or F296C sites have no chances to contact water molecules to be ionized and/or the expanded water-filled cavity containing HMTS molecules. In addition, some double mutants (A291W/A295C, A291W/F296C and S270I/F296C) also showed drastically reduced potentiations of the GABA-induced responses by hexanol and were not accessible to HMTS in the hexanol-bound state. The results indicate that S270 and A291 sites of alcohol-binding pocket might also play an important role in hexanol action. Taken together, the data of this study strongly support that S270 and A291 are critical sites for alcohol-binding, and alcohol-induced conformational changes. However, it is of interest to consider alternative hypotheses, including the idea that there are multiple alcohol-binding sites on the $GABA_A$ receptor, and/or in the lipid phase. It has recently been suggested that n-alkanols or anesthetics are solubilized in the bilayer, and this can

affect the bilayer packing free volume or membrane lateral pressures. Consequently, the conformational equilibrium of integral membrane proteins, such as ligand-gated ion channels or G-protein coupled receptors, can be affected in the membrane bilayer (Mitchell et al., 1996; Mohr et al., 2005). With respect to present experiments, if these possibilities were the main mechanisms for alcohol action, it would be expected that even the double mutants would react with HMTS in the alcohol-bound states. On the contrary, these results were not obtained. Although these alternative possibilities cannot be completely ruled out, the present data observed and other mutational studies (Lobo et al., 2004b; Lobo et al., 2004c; Mascia et al., 2000b; Mihic et al., 1997; Wick et al., 1998) suggest that there is an alcohol-binding pocket formed in part by TM 2 and 3 domains in $GABA_A$ and glycine receptors.

6.2 Alcohol Effect on function of $GABA_A$ receptor

There are several possible implications for the expansion of the water-filled cavity produced by GABA- or alcohol-induced conformational changes. First, if it is assumed that alcohols are binding in the existing water-filled cavity formed in part by TM3, then it appears that this binding promotes the expansion of this cavity or stabilizes a receptor conformational state in which the cavity is larger. The previous results have showed that the barbiturates, benzodiazepines or steroids increased $GABA_A$ receptor average channel open duration, opening frequency, channel mean open time or shifted frequency histograms of channel open times to longer times using single channel kinetic analysis, suggesting that open state of the $GABA_A$ receptor was stabilized by these potentiating modulators (MacDonald et al., 1989; Twyman and Macdonald, 1992; Twyman et al., 1989). Furthermore, it has been also suggested that channel gating and allosteric modulation by diazepam or propofol induce structural movements in the TM3 of $GABA_A$ receptor $\alpha 1$ subunit, and these conformational changes make the existing water-filled cavity around the upper TM3 segment to penetrate into the interior of the TM domain (Williams and Akabas, 1999; Williams and Akabas, 2000; Williams and Akabas, 2002). The results support a hypothesis that the modulators stabilize the open state of the receptor, and bind and interact with the water-filled cavity.

Recently, in the homologous glycine receptor, the channel gating has shown to increase the structural volume surrounding amino acids critical for alcohol action in TM2 and TM3 by conformational changes (Lobo et al., 2004b). The expansion of the water-filled cavity in TM3 domain of $GABA_A$ receptor $\alpha 1$ subunit may allow binding of additional molecules of alcohol and may also mimic some of the actions of GABA, thereby promoting channel opening. Furthermore, alcohol occupation of the water-filled cavity during gating would stabilize the structure of activated conformational state of the receptor and thus may lead to the alcohol-induced potentiation of GABA-induced currents (Figure 17).

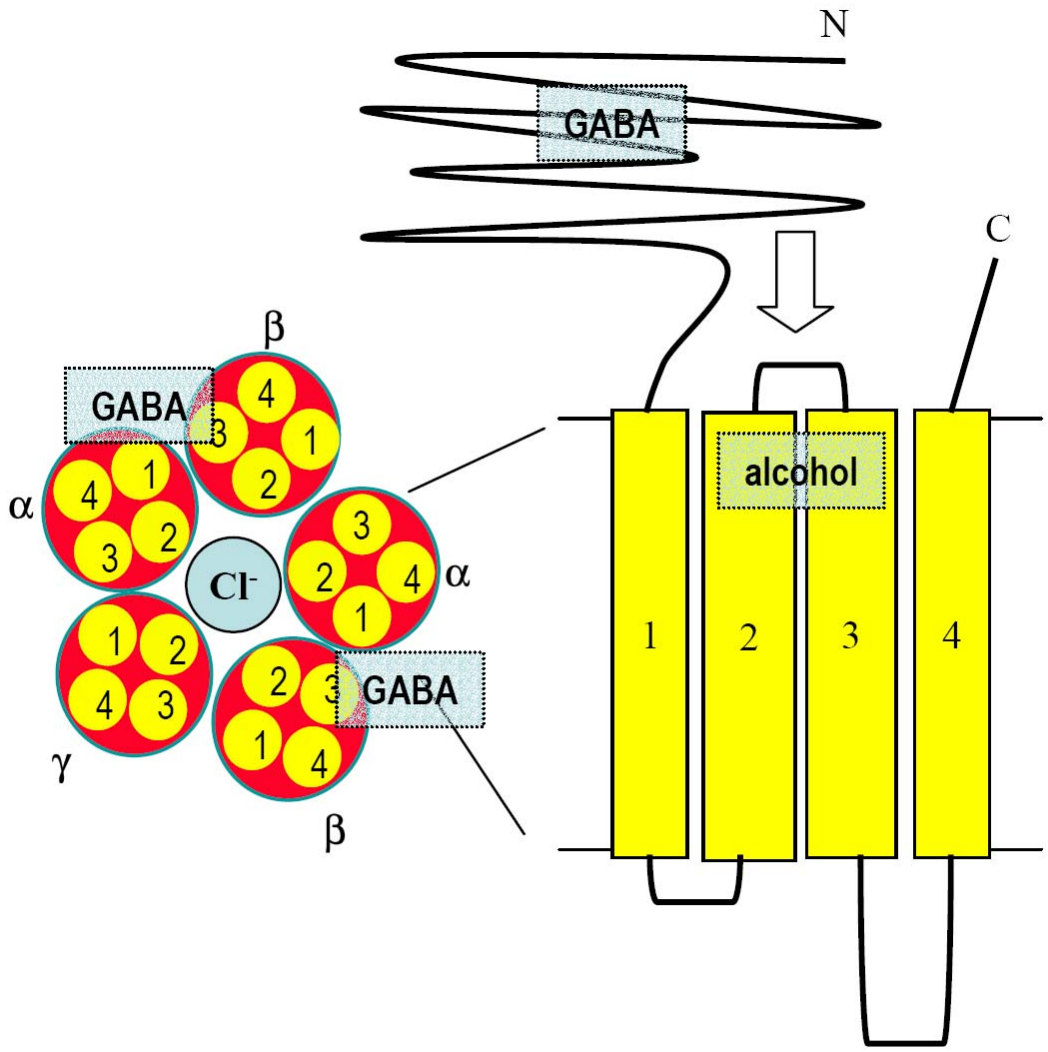

Figure 17: A schematic illustration of proposed ethanol action on stabilizing channel open-state. (1) GABA-binding to the interface between N-terminals of α and β subunits induces conformational changes, which are transduced to TM domains via TM2-3 loop, and then the channel opens, (2) alcohol binding to alcohol-binding pocket of α subunit stabilizes the open state and enhances the function of $GABA_A$ receptor. Four putative membrane-spanning helices are shown as cylinders. The arrow shows the transmission of extracelluar structural changes produced by binding of GABA to the N-terminal domain to TM 2.

A more general extension, it is of interest to note that the alcohol binding site in the Drosophila protein, LUSH, has some similarity to the putative alcohol binding site TM2/3 region of $GABA_A$ receptors (Kruse et al., 2003). This binding region in LUSH is a water-filled cavity which adopts multiple conformations when occupied by water, but is stabilized with limited movement when alcohols displace water (Kruse et al., 2003; Trudell and Harris, 2004). A variety of studies have shown that there is a binding cavity for alcohols and volatile anesthetics on GABA and glycine receptors (Jenkins et al., 2001; Koltchine et al., 1999; Mascia et al., 2000b; Mihic et al., 1997; Wick et al., 1998; Yamakura et al., 1999; Ye et al., 1998). Thus, the high flexibility observed for this region of TM3 in the present study may also allow for multiple conformations of the water-filled cavities and, perhaps, occupation of the cavity by alcohol selectively stabilizes substates which in turn stabilize the open state of the channel.

It should be noted that ligand-gated ion channels such as $GABA_A$ receptor undergoes rapidly conformational transitions through different affinity states from the resting to the open and then the desensitized states following ligand-binding (Changeux and Edelstein, 1998). However, in these surface accessibility experiments of MTS reagents, it is impossible to know in which state the MTS reaction is occurring in the presence of GABA. In the absence of detailed single channel kinetic analysis of the effects of covalent modification of MTS to the introduced cysteine, it is also very difficult to know whether the modification is affecting opening rates,

closing rates, or desensitization rates of the $GABA_A$ receptors during channel gating or alcohol modulation. Additionally, it will be necessary to investigate whether the covalent modification by MTS reagents to substituted cysteine residue affect channel open time or open probability by using single channel kinetic analysis. Furthermore, in the absence of high resolution structures of the $GABA_A$ receptors, it is not possible to provide structural explanations on how the covalent modifications by treatments of MTS reagents result in potentiation or inhibition of the function of the $GABA_A$ receptors. Further studies such as crystal structure of $GABA_A$ receptor with alcohol or single-channel analysis of alcohol effects on $GABA_A$ receptor will be necessary to assess exact effects of alcohol on $GABA_A$ receptor. Thus, the structural changes of water-filled cavity in the interior of the transmembrane domain during channel gating and modulation may provide a molecular basis for allosteric modulation of $GABA_A$ receptors by modulators such as alcohols.

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