Effects of Ethanol on Tonic GABA Currents in Cerebellar Granule Cells and Mammalian Cells Recombinantly Expressing GABA<sub>A</sub> Receptors

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ABSTRACT

The effects of ethanol on the GABA<sub>A</sub> receptors, which are regarded as one of the most important target sites of ethanol, are very controversial, ranging from potentiation to no effect. The 6 subunit-containing GABA<sub>A</sub> receptors expressed in Xenopus oocytes were recently reported to be potently augmented by ethanol. We performed patch-clamp experiments using the cerebellar granule cells and mammalian cells expressing recombinant GABA<sub>A</sub> receptors. In granule cells, the sensitivity to GABA increased from 7 to 11 days in vitro. Furosemide, an antagonist of α6-containing GABA<sub>A</sub> receptors, inhibited GABA-induced currents more potently at 11 to 14 days than at 7 days. Ethanol at 30 mM had either no effect or an inhibitory effect on currents induced by low concentrations of GABA in granule cells. On α4β2δ, α6β2δ, or α6β3δGABA<sub>A</sub> receptors expressed in Chinese hamster ovary cells, ethanol at 10, 30, and 100 mM had either no effect or an inhibitory effect on GABA currents. Ethanol inhibition of GABA<sub>A</sub> receptor was observed in all of the subunit combinations examined. In contrast, the perforated patch-clamp method to record the GABA currents revealed ethanol effects on the α6β2δ subunits ranging from slight potentiation to slight inhibition. Ethanol seems to exert a dual action on the GABA<sub>A</sub> receptors and the potentiating action may depend on intracellular milieu. Thus, the differences between the GABA<sub>A</sub> receptors expressed in mammalian host cells and those in Xenopus oocytes in the response to ethanol might be due to changes in intracellular components under patch-clamp conditions.

Ethanol is known to modulate the activity of a variety of neurotransmitters coupled receptors and ion channels. For example, ethanol potentiates the activity of GABA<sub>A</sub> receptors (Mihic, 1999), 5-hydroxytryptamine<sub>1A</sub> receptors (Loving, 1999), and neuronal nicotinic acetylcholine receptors (Narahashi et al., 1999). In contrast, ethanol has been reported to inhibit the activity of glutamate receptors (Woodward, 1999), voltage-gated calcium channels (Walter and Messing, 1999), and ATP-gated ion channels (Weight et al., 1999).

Whereas the importance of the GABA<sub>A</sub> receptors in the behavioral effects of ethanol is well documented (Pontieri et al., 1996; Koob et al., 1998), the ethanol modulation of GABA<sub>A</sub> receptor activity in vitro has been very controversial (Sapp and Yeh, 1998; Aguayo et al., 2002). Some investigators found the potentiation of GABA-induced currents (Mihic, 1999), whereas others did not find any effect of ethanol (White et al., 1990; Marszalec et al., 1994). A variety of explanations have been proposed for the controversial results on the effects of ethanol on the GABA<sub>A</sub> receptor. These include receptor subunit combinations (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), cell viability (Aguayo et al., 2002), cell type (Sapp and Yeh, 1998; Mori et al., 2000), and post-translational processing such as receptor phosphorylation (Aguayo et al., 2002), to mention a few.

Two lines of development have occurred recently with respect to the action of ethanol on GABA<sub>A</sub> receptors. One line is the subunit dependence of alcohol modulation of the GABA<sub>A</sub> receptor, and the other line is the significance of tonic GABA currents generated by extrasynaptic GABA<sub>A</sub> receptors. When recombinantly expressed in Xenopus oocytes, the α4β3δ and α6β3δ combinations of GABA<sub>A</sub> receptor subunits are highly sensitive to ethanol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004). These results suggest that ethanol, like many other GABA<sub>A</sub> receptor modulators, potentiates GABA currents in a subunit-specific manner. However, a recent consortium study from four laboratories (Borghese et al., 2006) has failed to confirm such claim. Tonic GABA-induced currents generated by low concentrations of...
GABA in the hippocampal neurons (Bai et al., 2001), the dentate gyrus granule cells (Wei et al., 2004), and cerebellar granule neurons (Hanchar et al., 2005) are potentiated by low concentrations of ethanol. The potentiating action of ethanol on the tonic current may be more important than its action on the phasic synaptic current, because the former current contributes more than two-thirds of the total charges in the GABAergic neuron (Hamann et al., 2002). However, 50 mM ethanol failed to potentiate the tonic current in the cerebellar granule neurons (Carta et al., 2004). Thus, the effects of a drug on neuroreceptors could be influenced by the host cells expressing the receptors (Sivilotti et al., 1997).

In the present study, we used GABA<sub>A</sub> receptor subunits recombinantly expressed in Chinese hamster ovary (CHO) cells to examine the acute effects of ethanol. These host cells are from mammals and might exhibit pharmacological properties different from *Xenopus* oocytes. Furthermore, we studied the ethanol actions on currents induced by low concentrations of GABA in cerebellar granule neurons, because tonic GABA currents were detected in these cells contained in slice preparations. Using the whole-cell patch-clamp method, we found that ethanol either inhibited or exerted no effect on GABA-induced currents, regardless of subunit combinations of GABA<sub>A</sub> receptors. Using the perforated patch-clamp method to record the whole-cell GABA currents from the α<sub>6</sub>β<sub>2</sub>δ receptors, we found that ethanol had a potentiating, an inhibitory, or no effect on the GABA-induced currents. Taking these results together, we propose that ethanol exerts a dual action on the GABA<sub>A</sub> receptors, an inhibitory and a potentiating action. The potentiating action is labile, being influenced by intracellular milieu that could be altered depending on the methods to record the GABA currents.

### Materials and Methods

#### Cell Preparations

**Culture of Cerebellar Granule Cells.** Cerebellar granule cells were cultured as described by Gallo et al. (1982) with some modifications. In brief, the cerebella were isolated from 6- to 8-day-old Sprague-Dawley rats, and cells were dispersed with trypsin and plated at a density of 1 x 10<sup>5</sup> cells/cm<sup>2</sup> on 12-mm Nunc coverglasses (Nalge Nunc, Naperville, IL), coated with 10 g/ml poly-L-lysine (Sigma-Aldrich, St. Louis, MO). The cultures were maintained up to 2 weeks in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, 20 U/ml penicillin, and 20 U/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C in air + CO<sub>2</sub> (90 + 10% by volume). Under this condition, more than 90% of the cells in these cultures are glutamatergic granule cells (Gallo et al., 1982).

**Transient Transfection in Chinese Hamster Ovary Cells.** CHO cells were also used as host cells for transfections of GABA<sub>A</sub> receptors, because HEK293 cells have been reported to have endogenous β subunits (Kirkness and Fraser, 1993; Fuchs et al., 1995; Ueno et al., 1996). CHO cells were transiently transfected using Lipofectamine. In brief, 5 μg of cDNA per 35-mm culture dish was used in a ratio of 2:2:1 (α/β/δ). Cells were used for patch-clamp experiments 24 to 48 h after transfection. The cells were also co-transfected with 0.5 μg of cDNA encoded with green fluorescence protein, which could be identified under the fluorescence microscope. Depending on the subunit combinations, the percentage of green fluorescent protein-positive cells that expressed functional GABA<sub>A</sub> receptors varied from 20 to 80%. We found that the β2-containing GABA<sub>A</sub> receptors were expressed better than the β3-containing receptors. The rat GABA<sub>A</sub> receptor subunit clones were provided by Dr. Steve Moss (University of Pennsylvania, Philadelphia, PA) (α<sub>6</sub>), Dr. Robert Macdonald (Vanderbilt University, Nashville, TN) (α<sub>4</sub> and δ), and Dr. Robert Pearce (University of Wisconsin, Madison, WI) (β<sub>2</sub>, β<sub>3</sub>, and γ<sub>2</sub>αs). The cDNA was subcloned into a mammalian expression vector. The pCMV vector was used for the expression of α<sub>4</sub> and δ subunits, the vector pCEP-4 was used to express β<sub>2</sub> and β<sub>3</sub> subunits, and the vector pRK5 was used to express α<sub>6</sub> subunit.

**Whole-Cell Current Recording**

Currents were recorded using both the conventional whole-cell and the perforated patch-clamp techniques at room temperature (22°C). Pipette electrodes were made from 0.5-mm (i.d.)-borosilicate glass capillary tubes and fire-polished. The electrodes had a resistance of 3 to 8 M<sub>Ω</sub> for cerebellar granule cells or CHO cells when filled with the pipette solution. The membrane potential was clamped at −80 mV, and a 5- to 10-min period was allowed following rupture of the membrane to equilibrate the cell interior with the pipette solution. Currents through the electrode were recorded by an Axopatch 200 amplifier (Molecular Devices, Sunnyvale, CA), and filtered at 2 kHz. Whole-cell current data are expressed as the mean ± S.D., and n represents the number of experiments.

In some experiments, amphotericin B perforated patch recording was used to minimize intracellular dialysis. Amphotericin B was first dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/ml to make a stock solution, which was in turn diluted with pipette solution to a final concentration of 200 μg/ml. The tip of the patch pipette was filled with the internal solution and then back-filled the rest of the patch pipette with the amphotericin B-containing solution. Even though the DMSO concentration in the patch pipette was around 1%, the DMSO concentration in the cell should have been very low, because DMSO was delivered from a point source. When the access resistance reached a steady level, recording was started. If the access resistance suddenly changed, indicating breaking of the membrane at the tip of the electrode, the cell was discarded.

### Solutions

The external solution for whole-cell patch-clamp experiments contained 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5.5 mM HEPES acid, 4.5 mM Na<sup>+</sup> HEPES, and 10 mM glucose. The pH was adjusted to 7.3 with HCl, and osmolality was adjusted to 300 mOsm by d-glucose. The internal solution contained 140 mM CsCl, 10 mM HEPES, 11 mM EGTA, 2 mM Mg<sup>2+</sup>·ATP, 0.2 mM Na<sup>+</sup> GTP, and 5.5 mM glucose. The pH was adjusted to 7.3 with CsOH, and osmolality was adjusted 300 mOsm by d-glucose. For current recording from granule cells, 130 mM CsCl and 10 mM tetraethylammonium-Chloride were substituted for 140 mM CsCl in internal solution. We have chosen internal solutions without added Ca<sup>2+</sup> ions, since we have been able to obtain stable recordings of GABA currents. We compared two solutions, one solution with and the other solution without 1 mM Ca<sup>2+</sup> and found no difference between them in terms of recording GABA currents.

### Chemicals

GABA (Sigma-Aldrich) was first dissolved in distilled water to make stock solutions. Furosemide and amphotericin B were obtained from Sigma-Aldrich, and 100% ethanol (USP grade) was obtained from Pharmco Product, Inc. (Brookfield, CT). Isoflurane, sevoflurane, and propofol were obtained from Anaquest (Madison, WI) (a division of BOC Inc.), Abbott Laboratories (Abbott Park, IL), and Sigma/RBI (Natick, MA), respectively. Furosemide and propofol were first dissolved in dimethyl sulfoxide before being added to the external solution (0.1% v/v). This concentration of dimethyl sulfoxide did not affect the GABA response. Test solutions for inhalation anesthetics were prepared immediately before experiments by diluting the saturated solution and were kept in air-free, closed glass bottles to prevent evaporation of the anesthetics.
Drug Application

GABA and other drugs were applied directly onto the cell by gravity using a fast delivery device consisting of multibarrel tubes connected to a perfusion fast-step switching system. The solution exchange time of this device was 10 to 20 ms. In some experiments, drugs were coapplied with GABA onto the cells.

Data Analysis

EC\textsubscript{50} values and their slope factors (Hill coefficients) were calculated using the following equation: $I = I_{\text{max}} C^n/(C^n + EC_{50}^n)$, where $I$ is the amplitude of GABA-induced current, $I_{\text{max}}$ the maximal current, $C$ the drug concentration, and $n$ is the Hill coefficient. The nonlinear regression analysis was carried out using the least-squares fitting method (SigmaPlot, version 8.0; SPSS Inc., Chicago, IL) with the aid of a microcomputer. Data are given as the mean ± S.D. with the numbers of experiments, $n$.

Results

The strategy for studying the effects of ethanol on GABA\textsubscript{A} receptors was to compare its effects on native neurons in cultures and on mammalian host cells in which various combinations of subunits were expressed. Cerebellar granule cells were chosen as native preparations for two reasons: first, the GABA\textsubscript{A} receptor underwent developmental changes in subunits during maturation in cultures; and second, the tonic current was easily discernible in cerebellar granule cells. To mimic the GABA tonic current, 300 nM GABA was used to apply to cells for a period of several minutes. This low GABA concentration was near the ambient level of GABA in vivo.

Developmental Changes in GABA-Induced Currents in Granule Cells. The sensitivity to GABA in cerebellar granule cells in primary culture is known to increase during development in vitro (Zheng et al., 1994). To characterize the GABA\textsubscript{A} receptors in cerebellar granule cells, whole-cell currents were estimated from the GABA dose-response curves shown in Granule Cells. The inverse benzodiazepine agonist Ro15-4513 is known to have a high affinity for the GABA\textsubscript{A} receptors. To confirm the existence of GABA\textsubscript{A} receptors in the cerebellar granule cells, the inhibitory effects of furosemide on GABA-induced currents were examined. Furosemide has been reported to be a selective antagonist of the GABA\textsubscript{A} receptors (Fish et al., 1997). Furosemide at 100 μM reversibly inhibited steady-state currents to 54.7 ± 5% ($n = 5$) of the control at 7 DIV and 29.8 ± 7% ($n = 5$) of the control at 14 DIV (Fig. 2). Because furosemide inhibits the GABA current more effectively at 14 DIV than at 7 DIV ($p < 0.05$), the α6 subunits-containing GABA\textsubscript{A} receptors increase during maturation in vitro.

Modulation by Ro15-4513 of GABA-Induced Currents. The inverse benzodiazepine agonist Ro15-4513 is known to have a high affinity for the α6-subunit-containing receptors and to antagonize the ataxic effects of ethanol. Although Ro15-4513 was reported to exert the inhibitory effects on GABA responses mediated by both α1- and α6-containing receptors (Kleingoor et al., 1991), its effects on the α6-subunit containing receptors remain controversial. Ro15-4513 has a very high affinity for the α6-containing GABA\textsubscript{A} receptors with a $K_d$ around tens of nanomolar ranges. Thus, at either 100 nM or 1 μM, Ro15-4513 should exert near maximal effects. Ro15-4513 was neither potent nor efficacious in inhibiting the tonic GABA current induced in cerebellar granule cells. Figure 3A shows the inhibitory effects of Ro15-4513 on GABA-induced currents when Ro15-4513 at 1 μM was coapplied with 300 nM GABA in a cerebellar granule cell (10–14 DIV) (inhibition to 68.8 ± 10.4% of the control; $n = 5$). GABA was applied onto the cell at low concentrations and for extended times to mimic the tonic currents observed in slices. The effects of Ro15-4513 on the tonic currents mediated by the α6β2δ and α6βδ5 receptors were also examined and compared with those of α6β2γ2s receptors because of the reported existence of δ-containing subunits in the extrasynaptic regions. Although Ro15-4513 at 100 nM potentiated currents induced by 100 nM GABA in the α6β2γ2s receptor (175 ± 10.7% of the control; $n = 4$), it inhibited GABA-induced currents in both α6β2δ and α6βδ5 receptors. Because α6β2δ and α6βδ5 receptors behaved similarly, their data were lumped together, resulting a reduction to 57.6 ± 7.1% of the control ($n = 6$) (Fig. 3). It is concluded that the opposite responses to Ro15-4513 between the α6β2δ and α6β2γ2 receptors are due to the presence of γ2 versus δ subunits.

The Effects of Ethanol on Currents Induced by a Low Concentration of GABA in Cerebellar Granule Cells. Tonic currents are generated in extrasynaptic receptors by low concentrations of transmitters, and occur in various regions of the brain (Bai et al., 2001; Caraica et al., 2004; Semyanov et al., 2004). Recent studies have shown that ethanol potentiates the tonic GABA currents recorded from the cerebellar (Hanchar et al., 2005) and hippocampal slice preparations (Wei et al., 2004).

To examine the effects of ethanol on tonic currents, nonde-sensitizing whole-cell currents were induced in cerebellar granule cells by prolonged applications of a low concentration of GABA. Figure 4 shows the effects of ethanol on the currents induced by 300 nM GABA in granule cells at 14 DIV.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Developmental changes in GABA-induced currents in cerebellar granule cells. A, currents evoked by rapid applications of GABA at concentrations ranging from 0.1 to 100 μM in neurons at 7 DIV (a) and 11 DIV (b). B, concentration-response relationships for GABA to activate neurons at 7 DIV (○) and 11 DIV (●). The EC\textsubscript{50} value decreased from 12.2 μM at 7 DIV to 3.1 μM at 11 DIV with little or no change in Hill coefficient (1.5 at 7 DIV and 1.25 at 11 DIV). The data points are the mean peak currents expressed as percentages of the control current, and the error bars are standard deviations ($n = 5$).}
\end{figure}
Ethanol at 30 mM inhibited the currents in two of six granule cells at 7 DIV and in four of seven granule cells at 10 to 14 DIV. Figure 4A illustrates an example of the lack of the effect of ethanol (Fig. 4Aa) and that of its inhibitory action (Fig. 4Ab) on the current induced by 300 nM GABA. The scattered bar graphs shown in Fig. 4A, b illustrate that ethanol has either no effect or inhibitory effect on GABA-induced currents. In no case was a potentiating action on GABA tonic currents seen. The mean amplitude of GABA currents in the presence of 30 mM ethanol was decreased to 86.5 ± 18.1% of the control (n = 6; p > 0.05, paired t test) at 7 DIV and 73.1 ± 26.7% (n = 7; p < 0.05, paired t test) at 10 to 14 DIV (Table 1).

### Table 1

<table>
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<tr>
<th></th>
<th>EC50</th>
<th>Hill Coefficient</th>
<th>Activation</th>
<th>30 nM Ethanol</th>
<th>100 nM Ethanol</th>
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<tr>
<td></td>
<td>μM</td>
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<tr>
<td>Granule cells, 7 DIV</td>
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<td></td>
<td>12.2 ± 0.16^a</td>
<td>1.52 ± 0.28</td>
<td>0.4</td>
<td>−13.5 ± 18.1</td>
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<tr>
<td>Granule cells, 11 to 14 DIV</td>
<td>3.10 ± 0.39^b</td>
<td>1.25 ± 0.11</td>
<td>5.1</td>
<td>26.9 ± 20.7</td>
<td>−20.0 ± 15.7^c</td>
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<tr>
<td>α6β2δ</td>
<td>0.24 ± 0.11</td>
<td>0.98 ± 0.11</td>
<td>55</td>
<td>10.0 ± 10.3</td>
<td>−20.0 ± 15.7^c</td>
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<tr>
<td>α6β3δ</td>
<td>0.41 ± 0.19</td>
<td>0.86 ± 0.05</td>
<td>43</td>
<td>3.42 ± 6.25</td>
<td>−17.6 ± 7.00^d</td>
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<tr>
<td>δ4β2δ</td>
<td>0.67 ± 0.22</td>
<td>1.02 ± 0.10</td>
<td>31</td>
<td>−15.0 ± 18.9</td>
<td>−26.0 ± 21.5^e</td>
</tr>
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* Percentages of receptor activation by 300 nM GABA as calculated by the equation under Data Analysis using the estimated EC50 values and Hill coefficients.

* These two values for EC50 values are significantly different (p < 0.05).

* The percentage of inhibition was significant at p < 0.05 compared with the control.

### Figures

**Fig. 2.** Furosemide block of tonic currents induced by 300 nM GABA in cerebellar granule cells at 7 and 14 DIV. A, representative traces showing that furosemide at 100 μM reversibly inhibited GABA-induced currents. B, furosemide at 100 μM inhibited currents to 54.7 ± 5% (n = 5) of the control at 7 DIV and to 29.3 ± 7% (n = 5) at 14 DIV. The data points are the mean peak currents expressed as percentages of the control, and the error bars are standard deviations (n = 5).

**Fig. 3.** Effects of Ro15-4513 on GABA-induced currents in cerebellar granule cells and CHO cells recombinantly expressing GABA<sub>A</sub> receptors. A, Ro15-4513 at 1 μM inhibited currents induced by 300 nM GABA. B, potentiating effects of Ro15-4513 on tonic currents induced by 100 nM GABA in the α6β2γ2<sub>3</sub> receptors (a) and inhibitory effects in the α6β3δ receptors (b). C, summary graph. Ro15-4513 at 1 μM inhibited tonic currents induced by 300 nM GABA in granule cells (68.8 ± 10.4% of the control; n = 5). Ro15-4513 at 100 nM potentiated tonic currents induced by 100 nM GABA in the α6β2γ2<sub>3</sub> receptor (175 ± 10.7% of the control; n = 4) and inhibited GABA-induced currents in the α6β2δ and α6β3δ receptors (57.6 ± 7.1% of the control; n = 6). The data points are the mean peak currents expressed as percentages of the control current, and the error bars are standard deviations.

### Discussion

To ascertain that there were no technical errors in the experimental procedures, we tested several other drugs that...
are known to modulate GABA<sub>A</sub> receptor currents on α4β2δ, α6β2δ, and α6β3δ subtypes expressed in CHO cells. Figure 6A shows a representative experiment obtained from the α6β2δ receptors. Potentiation of GABA-induced currents was consistently observed at clinically relevant concentrations of the inhalational anesthetics isoflurane (280 μM) (229.5 ± 16.0% of the control; n = 12) and sevoflurane (330 μM) (228.2 ± 20.7%; n = 5), and the intravenous anesthetic propofol (3 μM) (226.3 ± 26.3%; n = 8). In addition, current inhibition was observed by 100 μM furosemide (24.6 ± 5.2% of the control; n = 7) (Fig. 6B). Similar results were obtained in the α4β2δ and α6β3δ GABA<sub>A</sub> receptors as well (data not shown).

**Perforated Patch Experiments.** As described in the preceding sections, we never observed potentiation of GABA-induced currents by ethanol. This result is at variance with the ethanol potentiation of GABA currents in Xenopus oocytes recombiantinately expressing α4, α6, and δGABA receptor subunits (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). One possible reason for the discrepancy is that dialysis of intracellular micelles occurring under the whole-cell patch-clamp conditions prevents the ethanol-induced effect (Huang and Narahashi, 1997). In contrast, very little change in the intracellular milieu in oocytes is expected to occur in two-microelectrode voltage-clamp conditions. Because the role of phosphatases, protein kinase C, and protein kinase A is implicated in the action of ethanol (Morrow et al., 2004), changes in intracellular components under patch-clamp conditions may account for the differences in the responses to ethanol between our results and those using oocytes. To verify the validity of this hypothesis, we performed perforated patch-clamp experiments in which changes in intracellular components would be minimal.

Perforated patch experiments using amphotericin B were performed with the α6β2δ receptor expressed in CHO cells. Figure 7 shows three examples of changes in peak current amplitude during repeated 3-s applications of 300 nM GABA. These were given every 2 min during a continuous bath perfusion of 100 mM ethanol. Figure 7A depicts a potentiating action of 100 mM ethanol, and Fig. 7B illustrates an inhibitory action of ethanol. An example of the lack of effect of ethanol is shown in Fig. 7C. Thus, ethanol effects ranged from potentiation to inhibition. As a result, the mean amplitude of GABA currents in the presence of 100 mM ethanol was not significantly different from the control (Fig. 7D) (96.7 ± 10.9% of the control; n = 12; p > 0.05, paired t test).

Even though the effects of 100 mM ethanol on the GABA-
induced currents of the α6β2δ GABAA receptor were not significantly different between the whole-cell patch clamp methods (84.5 ± 25.7%) and the perforated patch-clamp method (96.7 ± 10.9%). It is instructive to examine the data distribution in detail using the skewness test and the box plot. First, we applied the skewness test to compare the two sets of data. The data from the whole-cell recordings were significantly (p < 0.05; n = 10) skewed toward positive with more values bunched together above the mean and with a long tail below the mean, whereas the data from the perforated patch clamp were not skewed. The box plot is useful to gain an overview of the spread of the data points and identify outliers (Fig. 8). The plot of the data obtained from the whole-cell patch-clamp technique also shows that the distribution is significantly skewed, because the median (98.5%) is not in the center of the box and differs greatly from the mean value (84.5%). In examining the data further among ten cells exposed to 100 mM ethanol, we have found that the GABA-induced currents in six cells remain essentially unchanged, and those in the remaining four cells are reduced to various degrees from few percentage points to 64%. Despite the large interquartile range, there is no outlier in the data. In contrast to the whole-cell patch-clamp data, the perforated patch-clamp results are not skewed as the median value (96.1%) is nearly identical to the mean value (96.7%). Among nine cells exposed to 100 mM ethanol, the GABA-induced currents are enhanced in two cells, not changed in four cells, and slightly decreased in three cells. The degree of the spread of data is reflected in the interquartile range of box plots and slightly decreased in three cells. The degree of the spread of data in whole-cell patch having a large interquartile range than those in the perforated patch. The box plot revealed that the conventional patch-clamp technique yields a larger variability of data. The wide spread of data are most probably attributed to the varying degree of changes in the intracellular milieu caused by dialysis with the patch pipette solution.

**Discussion**

Using the whole-cell patch-clamp technique to record the GABA-induced currents, we demonstrated that ethanol lacked a potentiating action on GABA-induced currents in cerebellar granule cells and in mammalian cell lines expressing the GABAA receptors. Ethanol had either no effect on GABA currents or exhibited an inhibitory action (Figs. 4 and 5) in both native neurons and cell lines. Using the perforated patch-clamp technique, we showed that ethanol effects ranged from potentiation to inhibition. The extent of inhibition was small. In agreement with previous studies (Feng and Macdonald, 2004), we were able to demonstrate that the volatile anesthetics isoflurane and sevoflurane, and the intravenous anesthetic propofol, consistently potentiated GABAA responses, whereas ethanol did not potentiate GABA currents recorded under the same conditions (Fig. 6). These results suggest that ethanol does not act like either volatile or intravenous anesthetics in potentiating GABAA responses. *Xenopus* oocytes, HEK cell line, and other cell lines such as CHO cells have been extensively used as expression hosts to study electrophysiological properties of transfected receptors. Differences in the kinetics and pharmacological profiles of GABAA receptors were reported to be dependent on host cells. Akk et al. (2004) reported a potent direct activation of the α4-containing GABAA receptors in HEK cells by pentobarbital, whereas Wafford et al. (1996) demonstrated a lack of direct activation of the α4β1γ2 receptors in oocytes. In a recent study, Mercik et al. (2003) reported different desensitization kinetics between the α1β2γ2s GABAA receptors expressed in HEK293 cells and those expressed in QT6 cells derived from fibroblasts. Thus, the lack of potentiating action of ethanol may depend on differences in intracellular factors of host cells (Harris and Mihic, 2004).

In the present study, cerebellar granule cells were identified morphologically in primary culture and their characteristics were examined. The apparent affinity of the receptor for GABA increased during development in vitro (Fig. 1) and furosemide, an antagonist of the α6 subunit-containing GABAA receptor, inhibited GABA-induced currents more potently at 14 DIV than at 7 DIV (Fig. 2). These findings might be related to an increase in the amount of GABAA receptors containing the α6 subunit during granule cell maturation in vitro (Zheng et al., 1994).

The GABAA receptor system has long been hypothesized to be a target of ethanol action. Despite numerous studies conducted so far, the effects of alcohol on the GABAA receptor function remain controversial (Aguayo et al., 2002). A recent study has shown that low concentrations of ethanol enhance tonic inhibition mediated by the β2 subunit-containing GABAA receptors in the hippocampal (Wei et al., 2004) and the cerebellar granule cells (Hanchar et al., 2005). In experiments using recombinantly expressed receptors, specific combinations of GABAA receptors (α4β3δ and α6β3δ) in *Xenopus* oocytes were found to be uniquely sensitive to the potentiating action of ethanol in a concentration-dependent manner (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). Borghese et al. (2006) reported that they had not been able to replicate the sensitivity of α4β3δ GABAA receptors to low concentrations of ethanol. It was also reported that a single amino acid substitution from arginine (R) to glutamine (Q) at amino acid position 100 in the α6 subunit increased alcohol sensitivity and ethanol-induced motor impairment (Hanchar et al., 2005). However, another group showed that ethanol did not increase the magnitude of tonic currents in the cerebellar granule cells (Carta et al., 2004). To further explore this issue, Valenzuela et al. (2005) compared the effects of ethanol on the tonic currents of granule cells in cerebellar slices from male inbred alcohol-intolerant rats (100Q geno-
GABA receptor subunits expressed in CHO cells and on the 
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select GABA receptor subtypes and the proportion of 
(Fig. 3). Ro15-4513 was not as potent in inhibiting GABA 
inhibited those mediated by the αβγ- and αβδ6 receptors (Fig. 3). Ro15-4513 was not as potent in inhibiting GABA 
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effect or no effect on α4, α6, and δ combinations of 
GABA receptor subunits expressed in CHO cells and on the 
GABA receptors of cerebellar granule cells when the whole- 
cell patch-clamp method was used to record the GABA-in- 
duced currents. This inhibitory action of ethanol is not de- 
pendent on the subunits tested in the present study. The 
300 nM GABA concentration was chosen because it 
was comparable to ambient GABA levels to simulate the 
tonic current. A question is raised as to whether the degree of 
activation of various GABA receptors would influence the 
ethanol effects because the effects of ethanol on GABA-
receptors are dependent on the concentration of GABA used 
to activate the receptors. Using the EC50 values and Hill coef- 
ficients for different receptor subtypes, we have calculated 
the percentages of receptor activation as shown in Table 1. The 
ethanol effect does not seem to be related to the degree of 
receptor activation. For example, 30 mM ethanol gave a 
similar inhibition of the GABA-induced current in both 7 DIV 
granule cells and in αγδ6-transfected CHO cells (−13.5 and 
−15%, respectively), despite that the degree of receptor acti- 
vation differed greatly (0.4 versus 31%).

The difference in the ethanol effect on the αβδ6GABA receptors between the whole-cell patch clamp and the perfor- 
atated patch clamp (Fig. 8) suggests that ethanol has a dual 
action on the receptor: a direct inhibitory action on the re- 
ceptor and an indirect potentiating action via the second 
 messenger system. In the majority of experiments in which 
ethanol was continuously present, the overall effects of eth- 
anol might represent the sum of the potentiating and inhib- 
itory actions. Depending on the dose-response relationship 
for these two actions, one might expect to see a bell-shape 
dose-response curve (Sundstrom-Poromaa et al., 2002).

We further propose that the intracellular components are 
responsible for variable effects of ethanol. Under the whole- 
cell patch-clamp conditions in small mammalian cells, the 
intracellular milieu will be dialyzed by the patch pipette 
solution, and the degree of solution exchanges may depend on 
pipette tip diameter. As a result, there are a variety of eth- 
anol effects on GABA receptors. In contrast, the two-micro- 
electrode voltage clamp used for Xenopus oocyte may mini- 
mize such variability. This may provide some explanation for 
the various results reported on the potentiating action of 
ethanol on various GABA receptors (Sapp and Yeh, 1998). 
Using the whole-cell patch clamp technique to examine the 
effects of ethanol on the GABA currents recorded from small 
cells to large cerebellar Purkinje cells, they found that 100 
mM ethanol increased the current by 40% in Purkinje cells, 
whereas it had no effect in cell lines. Further experiments 
should be designed to tease out the putative dual effect of 
ethanol.

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References

Aguayo LG, Peoples RW, Yeh HH, and Yevenes GE (2002) GABA receptors as 
molecular targets of ethanol action. Direct or indirect actions? Curr Top Med 
Pharmacol 2:809–885.

containing the αδ subunit by GABA and pentobarbital. J Pharmacol (Lond) 

Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF, and Orser BA (2001) 
Distinct functional and pharmacological properties of tonic and quantal inhibitory 
glutamatergic currents mediated by α1-aminobutyric acidA receptors in hippocampal 

Borghese CM, Storustovu SI, Ebert B, Herd MB, Belelli D, Lambert JJ, Marshall G, 
Wafford KA, and Harris RA (2006) The δ subunit of γ-aminobutyric acid type A 
receptors does not confer sensitivity to low concentrations of ethanol. J Pharmacol 
Exp Ther 316:1360–1368.

characterization of a novel cell line expressing human αβδ6 GABA receptors. Br J 

Carascales VB, Newell JG, You/TeK RK, Elliott EM, Rosahl TW, Wafford KA, Mac- 
Donald JF, and Orser BA (2004) Selective enhancement of tonic GABAergic 
inhibition in murine hippocampal neurons by low concentrations of the volatile 

Cara M, Mameli M, and Valenzuela CF (2004) Alcohol enhances GABAergic trans- 
mision to cerebellar granule cells via an increase in GOLI cell excitability. J 

Feng HJ and Macdonald RL (2004) Multiple action of propofol on αβδ6 and 

terminal domains in allosteric regulation of γ-aminobutyric acidA receptors. 


glutamate from cerebellar granule cells differentiating in culture. Proc Natl Acad 
Sci USA 79:7919–7923.

Hadingham KL, Garrett EM, Wafford KA, Bain C, Heavens RP, Sirinathsinghji DJ, 
and Whiting PJ (1996) Cloning of cDNA encoding the human 
α1-aminobutyric acid type A receptor α6 subunit and characterization of the pharmacology of α6- 

Hanchar HJ, Dodson PD, Olsen WA, Otis TH, and Walmer M (2005) Alcohol-induced 
motor impairment caused by increased extrasynaptic GABAergic receptor activity. 

Harris RA and Mihic SJ (2004) Alcohol and inhibitory receptors: unexpected speci- 


Hammann M, Rossi DJ, and Atwell D (2002) Tonic and spillover inhibition of granule 

Huang CS and Narahashi T (1997) The role of G proteins in the activity and mercury 

Carta M, Mameli M, and Valenzuela CF (2004) Alcohol enhances GABAergic trans- 
mision to cerebellar granule cells via an increase in GOLI cell excitability. J 

Feng HJ and Macdonald RL (2004) Multiple action of propofol on αβδ6 and αβδ6 

terminal domains in allosteric regulation of γ-aminobutyric acidA receptors. 


glutamate from cerebellar granule cells differentiating in culture. Proc Natl Acad 
Sci USA 79:7919–7923.

Hadingham KL, Garrett EM, Wafford KA, Bain C, Heavens RP, Sirinathsinghji DJ, 
and Whiting PJ (1996) Cloning of cDNA encoding the human 
α1-aminobutyric acid type A receptor α6 subunit and characterization of the pharmacology of α6- 

Hanchar HJ, Dodson PD, Olsen WA, Otis TH, and Walmer M (2005) Alcohol-induced 
motor impairment caused by increased extrasynaptic GABAergic receptor activity. 

Harris RA and Mihic SJ (2004) Alcohol and inhibitory receptors: unexpected speci- 


Hammann M, Rossi DJ, and Atwell D (2002) Tonic and spillover inhibition of granule 

Huang CS and Narahashi T (1997) The role of G proteins in the activity and mercury 

Carta M, Mameli M, and Valenzuela CF (2004) Alcohol enhances GABAergic trans- 
mision to cerebellar granule cells via an increase in GOLI cell excitability. J 


