Ethanol-GABA<sub>α</sub> Receptor Interactions: A Comparison between Cell Lines and Cerebellar Purkinje Cells<sup>1</sup>

DOUGLAS W. SAPP and HERMES H. YEH

Departments of Pharmacology (D.W.S., H.H.Y.), Neurology (H.H.Y.) and Program in Neuroscience (H.H.Y.), University of Connecticut Health Center, Farmington, Connecticut

Accepted for publication October 28, 1997 This paper is available online at http://www.jpet.org

ABSTRACT

This study compared the interaction between ethanol and γ-aminobutyric acid (GABA)-mediated current responses elicited in several immortalized cell lines and stably transfected cells, as well as in cultured and acutely dissociated rat cerebellar Purkinje cells. Only cell lines that were found previously to possess functional GABA<sub>α</sub> receptors were examined in this study. Under identical recording conditions, ethanol (10–200 mM) exerted no effect on GABA-induced currents in any of the cell lines or stably transfected cells tested in this study. However, GABA responses monitored in both primary culture and acutely dissociated Purkinje cells were significantly potentiated by ethanol (25 and 50 mM). Mouse pancreatic cells (RINm5F) were insensitive to both diazepam and ethanol suggesting the expression of a GABA<sub>α</sub> receptor isofrom lacking a γ subunit. Immortalized neuroblastoma IMR-32 cells displayed GABA responses that could be distinguished based on differential sensitivity to diazepam. However, none of the IMR-32 cells displayed GABA responses that were sensitive to modulation by ethanol. GABA responses in the stably transfected cell lines, PA3 (α1β1γ2<sup>a</sup>) and WSS-1 (α1β2γ2), were also unaffected by exposure to ethanol. In Purkinje cells acutely dissociated from the neonatal cerebellum, the ethanol-induced potentiation of GABA-induced current response could be observed before postnatal day 7, when only the γ2<sup>b</sup> but not the γ2<sup>a</sup> splice variant is expressed. This indicates that the γ2<sup>b</sup> subunit is not necessary for an ethanol-induced potentiation of GABA<sub>α</sub> receptor-mediated response to become manifest. In addition, the results point to inherent differences that should be taken into account in interpreting comparative data between native and recombinant GABA<sub>α</sub> receptors.

Considerable experimental attention in research on the central effects of ethanol has been directed toward examining its modulatory effects on the GABA<sub>α</sub> receptor complex. Early biochemical studies demonstrated an ethanol-induced potentiation of GABA-mediated <sup>36</sup>Cl<sup>-</sup> flux in synaptoneurosomes and microsomes derived from rat and mouse cerebral cortex (Allan and Harris, 1986; Mehta and Ticku, 1988; Suzdak <em>et al.</em>, 1986). This was largely corroborated by electrophysiological studies indicating that physiologically-relevant concentrations of ethanol potentiated GABA-mediated currents in cultured cerebral cortical and spinal cord neurons (Aguayo, 1990; Celentano <em>et al.</em>, 1988; Nishio and Narahashi, 1990; Reynolds and Prasad, 1991). Other electrophysiological studies, however, failed to observe modulatory effects of ethanol on neuronal responses to GABA (<em>Sigel et al.</em>, 1993; <em>Siggins et al.</em>, 1987; <em>White et al.</em>, 1990). One prevailing postulate to reconcile the diverse and apparently conflicting results related to sensitivity to modulation by ethanol takes into account the diversity of the known GABA<sub>α</sub> receptor subunits, of which 17 have been uncovered and classified into five families (reviewed in [Macdonald and Olsen, 1994; McKernan and Whiting, 1996; Sieghart 1995; Tyndale <em>et al.</em>, 1995; Yeh and Grigorenko, 1995]). The subunits display discrete yet overlapping patterns of distribution in the brain, and recombinant GABA<sub>α</sub> receptors of defined subunit combinations exhibit different yet predictable functional properties. These considerations, taken together, could account in theory for the observed modulation of GABA<sub>α</sub> receptors by ethanol among different types of neurons and brain regions.

Several studies have attempted to define the subunit composition of GABA<sub>α</sub> receptors sensitive to modulation by ethanol through the use of recombinant receptors assembled in a variety of expression systems. Thus, differences in sensitivity to ethanol have been found to depend on the α subunit isomere, because recombinant GABA<sub>α</sub> receptors expressing either the α1 or α6 subunit differed in rates of desensitization in response to co-application of GABA and ethanol (Marszalec <em>et al.</em>, 1994). Zolpidem, aside from its demonstrated high-affinity binding to Benzodiazepine type I receptors, also ap-

ABBREVIATIONS: GABA, γ-aminobutyric acid; PKC, protein kinase C; DMEM, Dulbecco’s modified eagle medium; PD, postnatal day; DMSO, dimethylsulfoxide; RT-PCR, reverse-transcriptase polymerase chain reaction; TBPS, t-butylbicylcophosphorothionate; <em>I</em><sub>max</sub>, maximal current; DZ, diazepam; EIOH, ethanol.
pears to be selective for receptors which are sensitive to potentiation by ethanol (Criswell et al., 1993).

The γ2 subunit exists as either a long or a short alternatively spliced variant (γ2L or γ2S) (Whiting et al., 1990). The γ2L splice variant contains in the intracellular loop between transmembrane domains TM3 and TM4 an additional 8-amino acid mini-exon that also harbors a consensus sequence for phosphorylation by PKC. In a series of studies using α1/β2/γ2-containing recombinant GABA<sub>A</sub> receptors expressed in Xenopus oocytes, a dependence on subunit specificity was demonstrated in that the ethanol-induced potentiation was observed only when the GABA<sub>A</sub> receptor assembly included the γ2L subunit (Wafford et al., 1991; Wafford and Whiting, 1992). However, a lack of effect by ethanol on either γ2L- or γ2S-containing recombinant GABA<sub>A</sub> has also been reported (Sigel et al., 1993), and the degree to which sensitivity to modulation by ethanol depends on the γ2L subunit remains an outstanding issue.

In this study we compared the effect of ethanol on whole-cell GABA-activated current responses under identical patch clamp electrophysiological recording conditions in two immortalized cell lines expressing endogenous functional GABA<sub>A</sub> receptors, two cell lines stably transfected with α1/β2/γ2 GABA<sub>A</sub> receptor subunits and in cerebellar Purkinje cells either maintained in long-term primary culture or after acute dissociation from the neonatal rat cerebellum. We chose to examine cerebellar Purkinje cells because they express GABA<sub>A</sub> receptor subunit profiles similar to those of the stably transfected cell lines examined (Laurie et al., 1992). We report that ethanol potentiated GABA-mediated current in Purkinje cells but had no effect on GABA responses recorded in any of the cell lines tested. Furthermore, an ethanol-induced potentiation of GABA responses in Purkinje cells could be observed at a time in development before the expression of the γ2L GABA<sub>A</sub> receptor subunit mRNA.

Materials and Methods

**Maintenance of cell lines in culture.** The RINm5F, IMR-32, WSS-1 and PA3/X25 cell lines were each maintained under conditions according to published reports (Hadingham et al., 1992; Noble et al., 1993; Tyndale et al., 1994; Wong et al., 1992). The RINm5F cell line was maintained in RPMI medium, the IMR-32 cell line in a minimum essential medium, and the WSS-1 cell line in DMEM. These media were supplemented with 10% fetal bovine serum and fungizone/streptomycin. The PA3 and X25 cell lines were cultured on 0.01% poly-D-lysine coated plates and maintained in DMEM-based serum-free growth medium supplemented with 0.01% poly-D-lysine and placed in an incubator for 1 hr to allow the cells to adhere. Before the seeding of cells, silicone droplets were attached to the periphery of each cover slip. Once the cells have adhered, the coverslips were placed inverted in 35-mm culture dishes containing a bed of cerebellar glial cells. Then 48 hr after plating, the serum-containing medium was replaced with a DMEM-based serum-free growth medium supplemented with B-27 nutrient mixture. The cultures were maintained in humidified 95% oxygen/5% carbon dioxide at 37°C and replenished with serum-free growth medium every 2 days. On selected days after plating, cerebellar cultures were fixed and processed immunohistochemically according to previously established procedures (Cheun and Yeh, 1992) to reveal calbindin-like immunoreactive cells. In vivo, only Purkinje cells express calbindin-like immunoreactivity in the cerebellum (Sequier et al., 1990). Calbindin-like immunoreactivity has also been used to identify Purkinje cells in culture (Brorson et al., 1991). Thus, calbindin-like immunoreactivity and cell morphology was used to identify Purkinje cells in culture for electrophysiological recording (see fig. 5A).

Acutely dissociated Purkinje cells were obtained from cerebella of PD-0 to -10 Sprague-Dawley rat pups as previously described (Cheun and Yeh, 1992). Briefly, rat pups were decapitated, the cerebellum was quickly removed, freed of the overlying dura, minced into approximately 1 mm³ pieces and incubated at 34°C in Earle's balanced salt solution containing papain (15 U/ml; Worthington, Freehold, NJ) for 60 min. The tissue was then treated with horse serum of ovomucoid to inactivate protease activity and washed several times with the bath solution used for electrophysiological recording. Gentle one-pass trituration of the tissue yielded a suspension of single cells that was then transferred to a 35-mm plastic Petri dish. The cells were allowed to adhere to the bottom of the dish, and acutely dissociated Purkinje cells were identified based on their morphology (Cheun and Yeh, 1992). All recordings were performed within 3 hr after acute dissociation.

**Electrophysiology.** Unless stated otherwise, whole-cell patch clamp recording of GABA-activated current responses were performed at room temperature in a bath solution containing (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 5 MgCl<sub>2</sub>, 5 HEPES, (pH 7.4). Some experiments involving PA3 and X25 cells employed bath solutions consisting of (in mM): 130 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 HEPES, 11 d-glucose, 13 sucrose (pH 7.2) in accordance with a previously published protocol (Harris et al., 1995a). Recording pipets were fire-polished to an input resistance of 5 to 10 MΩ. Seal formation and recordings were conducted using conventional whole-cell patch clamp procedures (Hamill et al., 1981), using an EPC-7 amplifier (Darmstadt, Germany). The recording solution contained (in mM): 140 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 HEPES, 3 Mg<sup>2+</sup> -ATP, 0.1 leupeptin (pH 7.4). Leupeptin was included to inhibit proteolysis and Mg<sup>2+</sup> -ATP was included to prevent possible rundown of the GABA activated response (Cheun et al., 1990). Membrane currents were amplified and filtered through a 4-pole Bessel filter. The analog signals were also monitored throughout the experiments using a Gould Brush 260 chart-recorder. GABA-activated currents were filtered, digitized and analyzed off-line using a data acquisition and analysis program (DATAQ/DATANAL, JPM Programming). Data were analyzed as previously described (Yeh and Kolb, 1997). Ethanol was determined to have a potentiating effect when there was a ≥20% increase in the GABA-ethanol response amplitude over mean control response.

**Preparation and delivery of drugs.** GABA, bicuculline methiodide and ethanol were dissolved bath solution. Dizepam was first dissolved in DMSO and then serially diluted in bath solution so that the final concentration of DMSO was <0.01%. Drugs were loaded into separate barrels of a multi-barrel pipet assembly and delivered by brief pulses of regulated pressure (∼2 PSI). The drug pipet assembly was mounted onto a micromanipulator so that it could be navigated under visual control to within 10 µm of the cell under study. This method facilitated brief focal applications of multiple test substances. One of the drug pipets within the multibarrel pipet assembly was routinely filled with bath solution that was applied continuously between epochs of drug application to clear drugs from...
the immediate vicinity of the cell and to control for possible mechanical artifacts due to bulk flow.

RT-PCR-based profiling of GABA<sub>A</sub> receptor subunit mRNAs. Candidate GABA<sub>A</sub> receptor mRNA profiles were obtained from the different cell line cultures as well as from selected single cells by means of an RT-PCR-based protocol (Yeh et al., 1996). For cultures, RNA was extracted with TRI reagent (Molecular Research Center), solubilized in RNase-free water and cDNA was synthesized by the addition of AMV reverse transcriptase (10 U) (Seikagaku, Ijamsville, MD), 1X first strand buffer, 2.5 mM dNTPs (Promega, Madison, WI), 10 ng oligo (dT) (Genosys, Woodlands, TX), RNasin inhibitor (20 U) and 10 mM dithiothreitol, incubated for 1 hr at 42°C. For single cells, first-strand cDNA was synthesized as above except that the oligonucleotide contained an oligo-dT region and the T7 RNA polymerase promoter. This facilitated one round of aRNA amplification using the T7 RNA polymerase before amplification by PCR (Eberwine et al., 1992). PCR amplification of the reversed transcribed cDNA template derived from either cultures or single cells was then performed in a solution containing thermal buffer, 2.5 mM MgCl<sub>2</sub>, 0.25 mM dNTPs, 2 U of Taq DNA polymerase (Promega), 50 pmol of primers, and 1 µl cDNA in a final volume of 20 µl. Samples were amplified for 30 or 40 cycles at 94°C for 45 sec, 60°C for 45 sec, 72°C for 1 min and a final extension was applied at 72°C for 7 min using a programmable thermocycler (Perkin-Elmer Cetus, Cupertino, CA). The amplified products were separated on a 2% agarose gel and visualized under UV illumination after staining with ethidium bromide. Whole-brain rat cDNA was used as positive control. Samples of reversed transcribed template to which no RNA was added served as negative control. Southern blot analysis, such as that illustrated in figure 6C, was performed as described previously (Yeh et al., 1996) using cDNA probes specific for the γ<sub>2a</sub> and γ<sub>2b</sub> GABA<sub>A</sub> receptor subunits.

Results

IMR-32

IMR-32 (human neuroblastoma) cells possess functional GABA<sub>A</sub> receptors, as revealed by TBPS binding autoradiography and GABA-stimulated chloride flux assays (Anderson et al., 1993). Western blot analysis demonstrated the presence of at least the α3 subunit (Noble et al., 1993), but the full complement of GABA<sub>A</sub> receptor subunits has yet to be characterized. In our study, we profiled the expression of GABA<sub>A</sub> receptor subunit mRNAs in cultured IMR-32 cells. An example of such an expression profile is illustrated in figure 1A. In addition to mRNA encoding the α3 subunit, α1, α4, β1, β3, γ2 and δ subunit mRNAs were also detected. Thus, numerous GABA<sub>A</sub> receptor subunit transcripts were found to be expressed by IMR-32 cells. However, pharmacological characterization of GABA-activated current response properties of individual IMR-32 cells suggests that not all IMR-32 cells express the full profile of subunits (D.W. Sapp and H.H. Yeh, manuscript in preparation). For example, individual IMR-32 cells could be shown to display GABA responses that were either sensitive or insensitive to potentiation by diazepam (fig. 2, A and B).

GABA (100 µM) delivered by regulated pressure induced whole-cell inward currents in IMR-32 cells held near their resting membrane potential. The GABA-activated current was blocked by 100 µM bicuculline methiodide (fig. 1B) and reversed at approximately 0 mV with equimolar [Cl<sup>−</sup>]<sub>in</sub> and [Cl<sup>−</sup>]<sub>out</sub> (fig. 1C). GABA was applied for variable lengths of time in order to derive an operationally defined range of response amplitudes that would be suitable for testing the effect of ethanol (fig. 1D, inset). With a 5-sec application of 100 µM GABA, the maximal response (I<sub>max</sub>) typically ranged between 200 to 300 pA (274 ± 31 pA, mean ± S.E.M.; n = 42). Figure 1D also illustrates data obtained from an IMR-32 cell in which amplitudes of the peak current response to GABA were plotted as a function of duration of agonist application. A “duration-response” relationship could be readily established. For each cell tested, the duration of agonist application was thus adjusted so as to produce current responses of constant amplitude approximately 20% of the I<sub>max</sub> in response to 100 µM GABA, and this was subsequently used to assess the modulatory effect of ethanol and diazepam on the same cell. As determined by data obtained from the 31 IMR-32 cells included in this study, the duration of GABA application ranged between 40 and 200 msec.

Ethanol at any concentration examined in this study (10–200 mM) had no effect on the GABA-mediated current responses in IMR-32 cells (fig. 2C). In figure 2A1 (top panel), the continuous penwriter record shows GABA-activated current responses monitored on-line before, during and after exposure to ethanol (100 mM) and diazepam (0.5 µM). The averaged digitized traces (fig. 2, A1 and A2) indicate that, although exposure to ethanol did not affect the amplitude of the GABA-activated current response, exposure to diazepam resulted in a reversible 81% potentiation of the GABA response in the same cell. Overall, in IMR-32 cells (n = 10) that could be shown to be sensitive to diazepam, the benzodiazepine-induced potentiation averaged 59 ± 16%, although ethanol exerted no effect in any of these cells. IMR-32 cells displaying GABA responses that were insensitive to modulation by diazepam were also encountered in this study (fig. 2B). Ethanol also did not affect the GABA responses in such cells (n = 21). The data obtained from diazepam-sensitive and -insensitive cells were pooled and are graphically summarized in figure 2C.

RINm5F

RINm5F cells display GABA-activated current responses that are blocked by bicuculline and zinc but are insensitive to potentiation by the benzodiazepines, suggesting the predominant expression of a GABA<sub>A</sub> receptor that lacks a γ subunit (Hales and Tyndale, 1994). Pressure application of 100 µM GABA (≥1 sec in duration) produced an I<sub>max</sub> of 64 ± 9 pA (mean ± S.E.M.; n = 8), similar to those reported previously (Hales and Tyndale, 1994). To assess the effect of ethanol, the duration of GABA application was adjusted for each cell so as to produce an approximately half-maximal level of the GABA-activated current response. The protocol for testing the effect of ethanol or diazepam on RINm5F responses to GABA was identical to that described above for testing IMR-32 cells. Neither a low (10 mM) nor a higher (100 mM) concentration of ethanol modulated the GABA-activated currents (fig. 3). The GABA-activated current responses monitored in RINm5F cells were also insensitive to potentiation by diazepam (fig. 3).

PA-3, X-25 and WSS-1. The PA-3 and X-25 cells are derived from a mouse fibroblast cell line that had been stably transfected with the α1/β1/γ2L and α1/β1 combinations of GABA<sub>A</sub> receptor subunits, respectively (Hadingham et al., 1992). In both <sup>36</sup>Cl<sup>−</sup> flux assays and electrophysiological studies (Harris et al., 1995a), GABA-mediated responses of PA3 cells, but not those of X-25 cells, were potentiated by 10 mM ethanol. In PA-3 cells maintained on a poly-l-lysine
substrate, the ethanol effect was observed at low GABA concentrations (3 and 10 $\mu$M). In our study, the interaction between ethanol and GABA-activated current responses was examined in PA-3 cells and X-25 cells under similar conditions using GABA at a concentration of 10 $\mu$M (fig. 4A). As was observed in every PA-3 cell tested ($n = 28$), a robust and readily reversible potentiation of the GABA response could be elicited upon exposure to diazepam but not to ethanol. In contrast, X-25 cells ($n = 12$) were neither sensitive to modulation by diazepam nor ethanol. Ethanol tested at a range from low to high concentrations (10, 30 and 100 mM) yielded the same results (fig. 4A).

Ethanol also did not modulate GABA responses monitored in the WSS-1 cell line that stably expresses functional $\alpha_1/\beta_2/\gamma_2$ recombinant GABA$_A$ receptors (Wong et al., 1992). An interaction between ethanol and GABA (10 $\mu$M) was tested in a total of 32 WSS-1 cells in the presence of ethanol at 10 mM ($n = 9$), 30 mM ($n = 11$) and 100 mM ($n = 12$). In 14 of the 32 cases, the effects of ethanol and diazepam on GABA-activated current responses were examined in the same cell. In each case, diazepam, but not ethanol, potentiated GABA responses ($192 \pm 18\%$ potentiation, $n = 14$; fig 4A). This is consistent with results obtained using the PA-3 cell line. Whether the long or short variant of $\gamma_2$ subunit was used for
transfecting WSS-1 cells was not specified in the original study (Wong et al., 1992).

The results of our RT/PCR-based mRNA profiling (fig. 4B) suggest the expression of the γ2<sub>S</sub> transcript in WSS-1 cells, that of the γ2<sub>L</sub> in PA-3 cells, both γ2<sub>S</sub> and γ2<sub>L</sub> mRNAs in IMR-32 cells, and an absence of any γ2 splice variant messages altogether in X-25 cells.

Cerebellar Purkinje cells

Cultured Purkinje cells. The outcome of an interaction between ethanol and GABA (25 μM) was examined in cultured Purkinje cells, identified based on size and morphology of calbindin-immunopositive profiles in long-term primary cultures derived from postnatal day 0 rat cerebellum (fig. 5A). The electrophysiological recordings were performed between 2 and 10 days in culture. In sharp contrast to the consistent lack of an effect in cell lines, ethanol (25 mM) potentiated the amplitude of the GABA-activated current responses elicited in cultured Purkinje cells. An ethanol-induced potentiation (34 ± 6%, mean ± S.E.M.; range = 20–42%) was seen in 10 of 14 cases (71%). In figure 5B, data derived from the 14 cells tested are summarized in the form of a scattergram and an example taken from an individual Purkinje cell is given in the inset.

Acute dissociated Purkinje cells. The ethanol-induced potentiation of GABA responses observed under long-term culture conditions was also found in Purkinje neurons acutely dissociated from neonatal cerebella (fig. 6A). Data from a total of 18 Purkinje cells, acutely dissociated from PD-3 (n = 4), PD-5 (n = 6), PD-7 (n = 6) and PD-10 (n = 2) rat cerebella, were included for analysis in this study (fig. 6B). The degree of potentiation induced by ethanol was highly variable and, given the limited sampling size for each postnatal age, it was not possible to attribute this variability to the age of the cerebellum from which the acutely dissociated Purkinje cells were derived. Acute exposure to ethanol resulted in a 39.0 ± 5.2% (mean ± S.E.M.) augmentation of the control GABA response.

An issue addressed was whether the expression of the γ2<sub>L</sub> subunit and its inclusion in the assembly of the GABA<sub>A</sub> receptor complex was required for conferring the ethanol-induced potentiation of the GABA response. Adult cerebellar Purkinje cells uniformly express both long and short variants of the γ2 GABA<sub>A</sub> receptor subunit mRNAs, in addition to those encoding the α1, β2, and β3 subunits (Gutierrez et al., 1994; Laurie et al., 1992). The presence of the γ2 subunit
mRNAs was confirmed in our study, insofar as combined patch clamp/RT-PCR in individual neurons revealed that Purkinje cells acutely dissociated from PD-7 or older cerebella with demonstrated sensitivity to GABA modulation by ethanol also expressed both the $g_{2L}$ and $g_{2S}$ subunit transcripts (fig. 6C). However, a striking and unexpected finding was that, although an ethanol-induced enhancement of GABA response could be observed in Purkinje cells before PD-7, the individual electrophysiologically-recorded cells expressed the $g_{2L}$ but not the $g_{2S}$ mRNA (fig. 6C).

Figure 6C focuses on the expression of the $g_{2S}$ and/or $g_{2L}$ messages in 3 PD-5 and 1 PD-7 acutely dissociated Purkinje cells, all of which had been verified electrophysiologically to display GABA responses that were sensitive to modulation by ethanol. The agarose gel (top panel) and Southern blot of the same gel probed with $g_{2S}$- and $g_{2L}$-specific oligonucleotides (lower panel) indicate that, while the $g_{2S}$ transcript...
could be readily detected in every Purkinje cell, that of the \( \gamma_2L \) was evident on PD-7 but not on PD-5. Thus, sensitivity to modulation by ethanol could be demonstrated in Purkinje cells that do not express the \( \gamma_2L \) mRNA.

**Discussion**

This study addresses two fundamental issues related to the relationship between GABA\( \Lambda \) receptor subunit combination and sensitivity to modulation by ethanol. The first issue relates to the discrepant results in the literature on ethanol-recombinant GABA\( \Lambda \) receptor interactions that have often been attributed to differences in experimental techniques and methods of analysis. To this end, ethanol-GABA\( \Lambda \) receptor interaction was examined under identical recording conditions in several cell lines and in cerebellar Purkinje cells. The second issue relates to whether there is a specific requirement of the \( \gamma_2 \) subunit in conferring ethanol sensitivity to native GABA\( \Lambda \) receptors, as has been reported for recombinant GABA\( \Lambda \) receptors, and this was examined in primary rat cerebellar cell cultures and in acutely-dissociated Purkinje cells.

**Subunit combination alone does not determine sensitivity to GABA\( \Lambda \) receptor modulation by ethanol.**

Taking the view that subunit composition is a critical determinant in the neuroactive nature of ethanol on the native GABA\( \Lambda \) receptor, sensitivity to modulation by ethanol can be expected to vary, depending on the GABA\( \Lambda \) receptor isomorph. Given the diversity of GABA\( \Lambda \) receptor subunits that can assemble to form functionally distinct receptor complexes, an intuitively logical approach toward resolving the issue of whether sensitivity to ethanol may depend on subunit composition has been to examine recombinant GABA\( \Lambda \) receptors of defined heterologous subunit combinations in expression systems. However, although several studies have reported subunit-specific dependence of the ethanol effect (Wafford et al., 1991), others have not (Marszalec et al., 1994; Korpi et al., 1995), and these discrepant outcomes have been commonly attributed to differences in the electrophysiological methods employed by individual laboratories. In this study, identical patch clamp recording conditions were employed to compare the effect of ethanol on GABA-mediated current responses in immortalized cells, stably transfected cells and rat cerebellar Purkinje cells either maintained in primary culture or isolated after acute dissociation. The PA-3 and WSS-1 cell lines express \( \alpha_1/\beta_1/\gamma_2 \) and \( \alpha_1/\beta_2/\gamma_2 \) recombinant GABA\( \Lambda \) receptors, respectively, similar to that expected to be expressed in cerebellar Purkinje cells because they express a limited set of \( \alpha_1/\beta_2/\beta_3/\gamma_2 \) transcripts (Hadingham et al., 1992). Our results clearly indicate that ethanol had no effect on GABA-mediated currents elicited in either the PA-3 or WSS-1 cell line, but potentiated GABA responses elicited in Purkinje cells. A systematic analysis of sensitivity to ethanol under varying concentrations of GABA was not performed in this study. Nonetheless, aside from this consideration, our results rule out differences in other electrophysiological recording conditions as confounding factors. Thus, we are led to conclude that subunit combination alone cannot account for sensitivity to modulation by ethanol.

The cell lines used in this study included immortalized cells that endogenously express functional GABA\( \Lambda \) receptors (RINm5F, IMR-32) as well as stably transfected cell lines that express recombinant GABA\( \Lambda \) receptors of defined subunit combinations (PA3, X25 and WSS-1). Ethanol exerted no modulatory effect on GABA-activated current responses monitored in RINm5F, IMR-32, PA3, X25 and WSS-1 cells. However, Diazepam markedly potentiated GABA responses elicited in the same PA3, WSS-1 and some of the IMR-32 cells that lacked sensitivity to ethanol, but did not modulate responses of either RINm5F or X25 cells to GABA. These results are consistent with \( \gamma \) subunit-containing GABA\( \Lambda \) receptors being sensitive to potentiation by diazepam (Knoflach et al., 1991; Pritchett et al., 1989; Ymer et al., 1990), but not necessarily by ethanol.

Two of the cell lines, IMR-32 and PA-3, were found to express mRNA transcripts encoding the \( \alpha_1 \) and \( \gamma_2L \) subunits. It was thus reasoned that the PA-3 and IMR-32 cell lines would be useful for addressing issues related to ethanol- and diazepam-GABA interactions. In agreement with a previous report employing \( [35S] \)TBPS binding and Cl\(^- \) uptake assays (Anderson et al., 1993), we found that the majority of IMR-32
cells displayed GABA responses that were insensitive to diazepam. However, a minor subpopulation of IMR-32 cells were sensitive to modulation by diazepam, suggesting that IMR-32 cells can express functional GABA<sub>A</sub> receptor isoforms that either contain or lack the γ2<sub>a</sub> subunit. Whether the individual IMR-32 cells examined electrophysiologically expressed exclusively either one of the two GABA<sub>A</sub> receptor isoforms, or both isoforms but to varying levels, could not be addressed in our study.

Previous Western blot characterization of the IMR-32 cell line revealed the presence of only the α<sub>6</sub> subunit protein; other GABA<sub>A</sub> receptor subunit proteins, including those for the α1 and γ2 subunits, were either not detected or not examined (Noble et al., 1993). It may be that the α1 and γ2<sub>a</sub> subunit proteins are expressed in a relatively small subpopulation of diazepam-sensitive IMR-32 cells that could be revealed by electrophysiological recording of individual cells but that their levels of expression are below the sensitivity of detection by Western blot analysis. In addition, the expression of the α3 subunit may predominate over that of α1, and IMR-32 cells may thus express primarily α3β2γ2<sub>_L,S</sub> GABA<sub>A</sub> receptors. In light of the potentiation of α1-containing GABA<sub>A</sub> receptors by zolpidem and its postulated link to ethanol (Criswell et al., 1993), α3-containing GABA<sub>A</sub> receptor isoforms may not be expected to be sensitive to potentiation by ethanol yet would be sensitive to diazepam. This could explain the lack of ethanol sensitivity in IMR-32 cells but falls short of explaining the same lack of ethanol sensitivity in PA-3 which stably express the α1/β1/γ2<sub>L</sub> subunit combination (Hadingham et al., 1992).

Ethanol modulation of native GABA<sub>A</sub> receptor function is independent of the expression of the γ2<sub>a</sub> subunit. In this study, ethanol-induced potentiation of GABA-mediated responses was manifested in acutely dissociated immature Purkinje cells prior to the detection of γ2<sub>a</sub> mRNA expression, indicating a lack of stringency for the expression of this subunit and the development of sensitivity to GABA<sub>A</sub> receptor modulation by ethanol. Consistent with this finding, ethanol potentiated GABA-mediated responses in cultured Purkinje cells as early as 2 days in vitro, well before the onset of γ2<sub>a</sub> expression (H.H. Yeh, unpublished observation).

In the adult state, cerebellar Purkinje cells express a relatively simple combination of α1, β2, β3 and γ2 receptor subunits as determined by in situ hybridization (Laurie et al., 1992) and immunocytochemistry (Gutierrez et al., 1994). Both long and short splice variants of the γ2 subunit are present in approximately equal levels in the adult but, before postnatal day 7 during development, only the expression of the γ2<sub>a</sub> subunit is detected (Bovolin et al., 1992; Wang and Burt, 1991). Although the presence of a given GABA<sub>A</sub> receptor subunit mRNA transcript does not necessarily imply translation and functional assembly of the encoded subunit protein, the absence of a given subunit mRNA transcript argues strongly against the presence of the encoded protein. Thus, our observation that ethanol potentiates GABA-activated current responses in immature Purkinje cells, when only the γ2<sub>a</sub> subunit mRNA can be detected, provides compelling evidence against an absolute requirement of the γ2<sub>a</sub> subunit in conferring sensitivity to native GABA<sub>A</sub> receptor modulation by ethanol. This information would not have been revealed without using the combination of patch clamp recording and single cell RT-PCR. Indeed, other studies have failed to document that ethanol differentially modulates receptors containing either the γ2<sub>a</sub> or γ2<sub>_R</sub> subunit. Sigel et al. (1993) reported that recombinant GABA<sub>A</sub> receptors expressed in Xenopus oocytes consisting of either the α1β2γ2<sub>_L</sub> or α1β2γ2<sub>_R</sub> subunits were equally potentiated by ethanol. Ethanol also failed to differentiate [35S]TBPS binding between γ2<sub>a</sub> and γ2<sub>_R</sub>-containing recombinant receptors coexpressing either the α1β2 or α6β2 subunits (Korpi et al., 1995).

The WSS-1 cell line was generated by stably transfecting HEK-293 cells with the α1/β2/γ2 combination of GABA<sub>A</sub> receptor subunits, although the spliced form of the γ2 transcript used for transfection was not defined (Wong et al., 1992). Our RT-PCR-based mRNA profiling of the electrophysiologically recorded WSS-1 cells revealed the expression of the γ2<sub>_R</sub> transcript. This GABA<sub>A</sub> receptor subunit profile thus resembles that found in immature Purkinje cells before postnatal day 7. However, it should be noted that tests of ethanol-GABA interaction in WSS-1 and Purkinje cells yielded contrasting outcomes.

Overall, the results of this study demonstrate that there are important inherent differences between GABA<sub>A</sub> receptor subunits in cell lines and those expressed in neurons, and that rules governing the relationship between subunit specificity and ethanol sensitivity in recombinant receptors may not be directly pertinent to those governing native GABA<sub>A</sub> receptors. Indeed, it would not be surprising if post-translational processes involved in the assembly of GABA<sub>A</sub> receptor subunits differ among cell lines and neurons in culture or in situ. As a specific example, the state of phosphorylation of the GABA<sub>A</sub> receptor may be a critical factor in assessing the outcome of an interaction with ethanol. Phosphorylation triggered by the activation of second messenger cascades has been demonstrated to play a role in mediating ethanol-induced potentiation of GABA responses in hippocampal (Weiner et al., 1997) and cerebellar Purkinje cells (Freund and Palmer, 1997). Also, a PKC-null mutant mouse line has been reported to exhibit decreased behavioral sensitivity to ethanol (Harris et al., 1995b). GABA-mediated Cl<sup>−</sup> uptake in synaptoneurosomes prepared from control mice but not from PKC-null mutant mice was potentiated by ethanol. Along this line of thought, data derived from cell lines may determine the subunit dependence or specificity underlying sensitivity to modulation by ethanol should be interpreted with caution in light of their applicability towards understanding interactions between ethanol and native GABA<sub>A</sub> receptors.

Acknowledgments

The authors thank Dr. Paul Whiting for the generous gift of the PA3 and X25 cell lines and Dr. E. V. Grigorenko for assistance in obtaining the data illustrated in figure 6.

References


Broersen JR, Bleakman D, Gibbons SJ and Miller RJ (1991) The properties of


Send reprint requests to: Douglas W. Sapp, Department of Pharmacology, MC6125, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030.