Ethanol potently and competitively inhibits binding of the alcohol antagonist Ro15-4513 to $\alpha_{4/6}\beta_3\delta$ GABA$_A$ receptors

H. Jacob Hanchar*†, Panida Chutsrinopkun†‡, Pratap Meera§, Porntip Supavilai‡, Werner Sieghart¶, Martin Wallner*‖, and Richard W. Olsen*‖

Departments of *Molecular and Medical Pharmacology and §Neurobiology, University of California, Los Angeles, CA 90095; ‡Department of Pharmacology, Faculty of Science, Mahidol University, Bangkok 10 400, Thailand; and ¶Center for Brain Research, Division of Biochemistry and Molecular Biology and Section of Biochemical Psychiatry, Medical University of Vienna, A-1090 Vienna, Austria

Edited by Floyd E. Bloom, The Scripps Research Institute, La Jolla, CA, and approved February 10, 2006 (received for review November 16, 2005)

Although GABA$_A$ receptors have long been implicated in mediating ethanol (EtOH) actions, receptors containing the ''nonsynaptic'' δ subunit only recently have been shown to be uniquely sensitive to EtOH. Here, we show that δ subunit-containing receptors bind the imidazo-benzodiazepines (BZs) flumazenil and Ro15-4513 with high affinity (K_d < 10 nM), contrary to the widely held belief that these receptors are insensitive to BZs. In immunopurified native cerebellar and recombinant δ subunit-containing receptors, binding of the alcohol antagonist [^{3}H]Ro15-4513 is inhibited by low concentrations of EtOH ($K_i \approx$ 8 mM). Also, Ro15-4513 binding is inhibited by BZ-site ligands that have been shown to reverse the behavioral alcohol antagonism of Ro15-4513 (i.e., flumazenil, β-carbolinecarboxylate ethyl ester (β-CCE), and *N*-methyl-β-carboline-3-carboxamide (FG7142), but not including any classical BZ agonists like diazepam). Experiments that were designed to distinguish between a competitive and allosteric mechanism suggest that EtOH and Ro15-4513 occupy a mutually exclusive binding site. The fact that only Ro15-4513, but not flumazenil, can inhibit the EtOH effect, and that Ro15-4513 differs from flumazenil by only a single group in the molecule (an azido group at the C7 position of the BZ ring) suggest that this azido group in Ro15-4513 might be the area that overlaps with the alcohol-binding site. Our findings, combined with previous observations that Ro15-4513 is a behavioral alcohol antagonist, suggest that many of the behavioral effects of EtOH at relevant physiological concentrations are mediated by EtOH/Ro15-4513-sensitive GABA$_A$ receptors.

alcohol receptor | flumazenil | β-carbolines | extrasynaptic GABA$_A$ receptors

Although many proteins show changes in their function at very high alcohol concentrations (>50 mM), the molecular basis for behavioral alcohol effects at low to moderately intoxicating doses experienced during social alcohol consumption remains elusive (1). GABA$_A$ receptors (GABA$_A$Rs) and the inhibitory GABAergic system have long been suspected to be targets for acute alcohol effects (2–4). For example, the GABA$_A$R agonist muscimol potentiates the sedative actions of alcohol, whereas the opposite effect, a reduction of ethanol (EtOH)-produced sedation, is detected with the GABA$_A$R blockers picrotoxin and bicuculline (5). Although most GABA$_A$R subunit combinations can be activated by high (anesthetic) alcohol concentrations (6), only very specific GABA$_A$R subunit combinations (containing the δ as well as the β_3 subunit) exhibit dose dependencies that mirror blood alcohol levels that are associated with mild to moderate intoxication in humans (7, 8) ($\approx$3–30 mM, because the legal drinking limit is 17 mM or 0.08%). GABA$_A$Rs containing the δ subunit are located either outside (9) or in the perimeter of (10) synapses but not in the subsynaptic membrane, and they give rise to a persistent (tonic) GABA current (11) that is enhanced by low alcohol concentrations (12–14). However, there is controversy because Carta *et al.* (14) concluded that the EtOH-induced increase in tonic current is due to increased GABA release rather than a postsynaptic effect. Also, for reasons on which we can only speculate, a recent article by Borghese *et al.* (15) states that effects of low dose EtOH on δ subunit-containing receptors could not be observed, in particular with human $\alpha_4\beta_3\delta$ GABA$_A$R clones expressed in oocytes and cell lines.

EtOH pharmacology shares many characteristics with allosteric activators of GABA$_A$Rs (loosely referred to as GABA$_A$R agonists), such as benzodiazepines (BZs) (5). Additional evidence for a link between EtOH and BZ actions on GABA$_A$R comes from the surprising finding that a mutation in the α_6 subunit (α_6-R100Q), which was identified in alcohol-nontolerant and -nonpreferring rats in refs. 16 and 17, leads to receptors with increased alcohol sensitivity in recombinant expression (in cerebellar granule cell neurons) and to alcohol-hypersensitive animals in behavioral studies (12). Histidine residues at positions that are homologous to the α_6-R100 residue (which affects EtOH sensitivity in $\alpha_6\beta_3\delta$ GABA$_A$Rs) are critical for high-affinity binding of classical BZ agonists at the interface between α and γ_2 subunits.

It has been thought that the ''extrasynaptic'' GABA$_A$R δ subunit, which presumably takes the position of the γ_2 subunit in functional pentameric GABA$_A$Rs, renders receptors insensitive to BZs (18). Also, most δ subunits are found to be associated with α_4 and α_6 subunits that differ from other GABA$_A$R α subunits at a critical position (a histidine in $\alpha_{1,2,3,5}$ replaced by an arginine in $\alpha_{4,6}$) that makes $\alpha_{4/6}\beta_x\gamma_2$ receptors insensitive to classical BZ agonists such as diazepam (DZ) and flunitrazepam (19, 20). However, Arg-100 (WT) in α_4 and α_6 receptors still allows high-affinity binding of the imidazo-BZs Ro15-4513 and flumazenil (in $\alpha_x\beta_x\gamma_2$ GABA$_A$Rs). In functional receptor assays, Ro15-4513 is a weak partial inverse agonist (i.e., it leads to a slight reduction in GABA$_A$R activity) on the most abundant GABA$_A$R subtypes in the brain (20). Ro15-4513 is a partial agonist (i.e., it enhances GABA action, but less than DZ even at saturating concentrations) on the α_4 and α_6 receptors (with β and γ_2 subunits), whereas flumazenil is essentially silent in functional

Conflict of interest statement: M.W., R.W.O., and H.J.H. have filed a U.S. Provisional Patent Application, Serial No. 60/693,844.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: GABA$_A$R, GABA$_A$ receptor; HEK, human embryonic kidney; BZ, benzodiazepine; EtOH, ethanol; IP, immunoprecipitated/immunoprecipitation; β-CCE, β-carboline-3-carboxyethyl ester; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; DZ, diazepam; DZ-IS, DZ-insensitive; FG7142, *N*-methyl-β-carboline-3-carboxamide.

See Commentary on page 8307.

†H.J.H. and P.C. contributed equally to this work.

‖To whom correspondence may be addressed at: Department of Molecular and Medical Pharmacology, University of California, Room 23-120 CHS, Charles Young Drive South, Los Angeles, CA 90095-1735. E-mail: mwallner@mednet.ucla.edu or rolsen@mednet.ucla.edu.

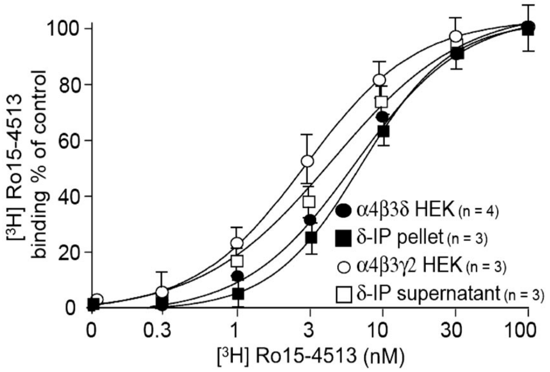

Fig. 1. EtOH-sensitive $\alpha_{4/6}\beta_3\delta$ $GABA_A$Rs have a high-affinity Ro15-4513-binding site. [^{3}H]Ro15-4513 saturation binding in native immuno-purified (δ-IP pellet) and δ-depleted (δ-IP supernatant) cerebellar $GABA_A$R fractions and to recombinant $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2$ $GABA_A$Rs receptor expressed in the HEK 293T cell line.

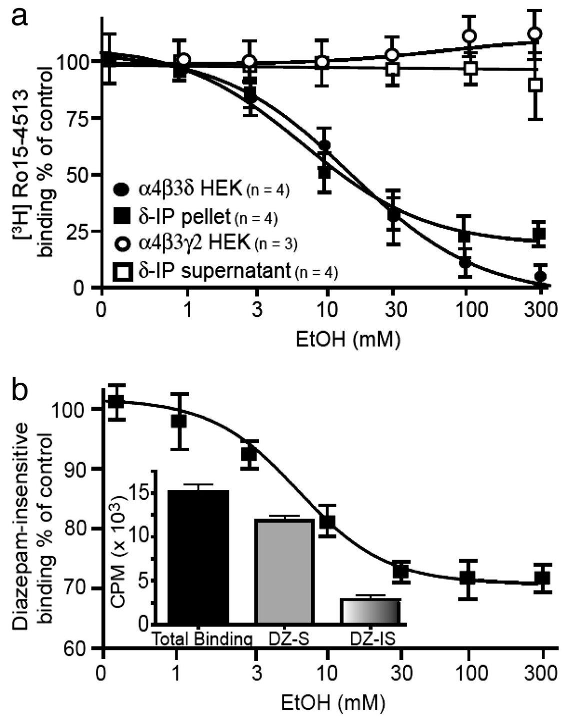

Fig. 2. Alcohol receptor binding assay. EtOH-displaceable high-affinity [^{3}H]Ro15-4513 binding to native and recombinant δ subunit-containing $GABA_A$R. (*a*) [^{3}H]Ro15-4513 binding is inhibited by low concentrations of EtOH. Receptors were equilibrated with 10 nM [^{3}H]Ro15-4513 and varying EtOH concentrations. We tested native immunopurified (δ-IP pellet) and δ-depleted (δ-IP supernatant) cerebellar $GABA_A$R fractions, and recombinant $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_2$ $GABA_A$Rs expressed in the HEK 293T cell line. (*b*) Approximately one-third of the DZ-IS [^{3}H]Ro15-4513 binding to cow cerebellar $GABA_A$Rs is antagonized by low EtOH concentrations. (*Inset*) Total, 10 μM DZ-sensitive, and DZ-IS 30 nM [^{3}H]Ro15-4513 binding to cow cerebellar membranes.

assays. Interestingly, Ro15-4513, but not other inverse agonists [like the β-carbolines β-carboline-3-carboxyethyl ester (β-CCE) and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM)], has been shown to have fairly dramatic alcohol-antagonist actions, reported in various mammals; in particular, alcohol effects at lower doses can be reversed almost completely by Ro15-4513 (3, 21–25).

Here, we show that alcohol-sensitive $\alpha_{4/6}\beta_3\delta$ $GABA_A$Rs bind [^{3}H]Ro15-4513 with high affinity, and that this binding is inhibited dose-dependently by low doses of EtOH. This result is consistent with the observation that Ro15-4513 potently inhibits EtOH enhancement of functional $\alpha_{4/6}\beta_3\delta$ $GABA_A$Rs (45). The Ro15-4513/EtOH antagonism appears to be competitive. Importantly, our data explain the puzzling observation that Ro15-4513 is a behavioral alcohol antagonist and suggest that EtOH/Ro15-4513-sensitive $GABA_A$Rs are important mediators of alcohol actions that are experienced during moderate social drinking.

Results

Alcohol Antagonist Ro15-4513 Binds to δ Subunit-Containing $GABA_A$Rs. Based on the observations that δ subunit-containing receptors are uniquely sensitive to EtOH (7), and that a BZ-site single-nucleotide polymorphism (α_6-R100Q) increases alcohol sensitivity in $\alpha_6\beta_3\delta$ receptors (12), we decided to investigate whether native and recombinant δ subunit-containing receptors could bind the behavioral alcohol antagonist Ro15-4513, which is commercially available as a tritiated radioligand.

To study native receptors, we immunopurified δ subunit-containing $GABA_A$Rs from cow cerebellum. The most likely subunit composition of these immunopurified receptors is $\alpha_6\beta\delta$. Based on the high EtOH sensitivity of cerebellar tonic currents and the increase in alcohol sensitivity observed with the α_6-R100Q allele, it seems likely that most of these receptors contain the β_3 subunit (12). In addition to native receptors, we also used recombinant $\alpha_4\beta_3\delta$ receptors expressed in eukaryotic [human embryonic kidney (HEK) 293T] cells.

Saturation-binding experiments showed that both receptor preparations exhibited high-affinity [^{3}H]Ro15-4513 binding (K_d = 7.5 ± 0.1 nM, on recombinant $\alpha_4\beta_3\delta$ receptors) that is in the same range as that found for recombinant $\alpha_4\beta_3\gamma_2$ receptors (K_d = 2.9 ± 0.7 nM; Fig. 1). K_d values were 7.0 ± 0.4 nM [for [^{3}H]Ro15-4513 binding for native δ-immunoprecipitated (IP) pellet] and 5.7 ± 0.6 nM [in the immunodepleted supernatant (δ-IP supernatant) containing native γ_2-containing receptors].

EtOH Inhibits [^{3}H]Ro15-4513 Binding to δ Subunit-Containing $GABA_A$Rs. Inspired by the finding that these alcohol-sensitive receptors bind the alcohol antagonist Ro15-4513 with high affinity, we investigated whether [^{3}H]Ro15-4513 binding to δ subunit-containing $GABA_A$R can be inhibited by EtOH. Fig. 2*a* shows that [^{3}H]Ro15-4513 binding to native cerebellar δ subunit immunopurified receptors (δ-IP pellet), as well as to recombinant $\alpha_4\beta_3\delta$ receptors, was dose-dependently inhibited by 3–300 mM EtOH ($IC_{50} \approx 12$ mM). In marked contrast, [^{3}H]Ro15-4513 binding to $\alpha_4\beta_3\gamma_2$ and the δ subunit immunodepleted supernatant (with γ_2 subunit-containing receptors) was essentially insensitive to EtOH, even at very high concentrations. However, in marked contrast to Ro15-4513 and despite its close structural similarity with it, [^{3}H]flumazenil (see Fig. 5) binding to recombinant $\alpha_4\beta_3\delta$ receptors was not inhibited by ≤300 mM EtOH (data not shown).

Alcohol Inhibits a Fraction (≈6%) of Cerebellar [^{3}H]Ro15-4513 Binding. Binding of [^{3}H]Ro15-4513 to $GABA_A$Rs in the cerebellum has been considered to include sites that are sensitive to classical BZ agonists (DZ-S binding) on $\alpha_1\beta\gamma_2$ isoforms and DZ-insensitive (DZ-IS) binding sites observed in the presence of 10–100 μM DZ. The latter are thought to be largely composed of $\alpha_6\beta\gamma_2$ subunits (26–28). Based on our finding that δ subunit-containing $GABA_A$Rs bind [^{3}H]Ro15-4513 with high affinity, and that this binding is sensitive to displacement by EtOH, we reasoned that a fraction of the DZ-IS binding sites in brain might be due to δ subunit-containing receptors. In agreement with previous stud-

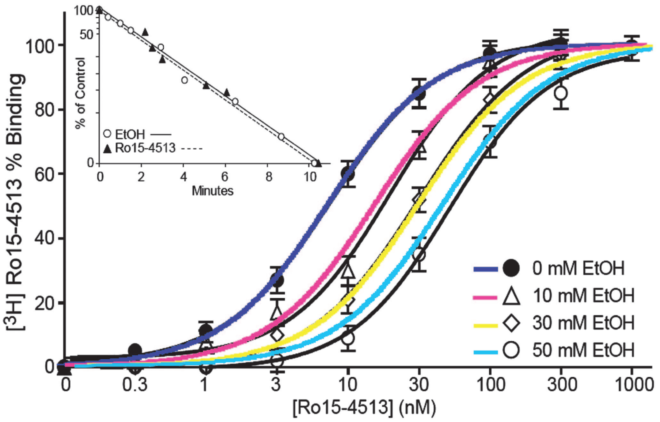

Fig. 3. Test for competitive antagonism between EtOH and Ro15-4513. Ro15-4513 saturation binding was performed in the presence of 10, 30, and 50 mM EtOH. Individual curves were fitted with the Hill equation (shown as black curves). Colored curves are derived from a simultaneous least-square fit of the entire data set by using the following equation: $[^3H]Ro15\text{-}4513 = (f/1-f)^{1/h} \times K_d \times (1 + [EtOH]/K_{d\ EtOH})$. $[^3H]$Ro15-4513 is the concentration of $[^3H]$Ro15-4513 to reach a fractional occupancy (f), h is the Hill coefficient, K_d is the dissociation constant for $[^3H]$Ro15-4513, and the term $(1 + [EtOH]/K_{d\ EtOH})$ is derived from the Schild–Gaddum equation and describes the decreased receptor occupancy by Ro15-4513 in the presence of the proposed competitive antagonist EtOH, where [EtOH] is the EtOH concentration, and $K_{d\ EtOH}$ is the dissociation constant for EtOH. The best fit of curves was obtained with a Hill coefficient of 1.1, a $K_{d\ EtOH}$ of 8.0 mM, and a K_d for Ro15-4513 of 7.5 nM. (*Inset*) Dissociation of $[^3H]$Ro15-4513 from $\alpha_4\beta_3\delta$ receptors equilibrated with 5 nM $[^3H]$Ro15-4513. The unbinding rate of $[^3H]$Ro15-4513 was measured by adding excess (1 μM) cold Ro15-4513 followed by rapid filtration after ≈0.5, 1, 2, 5, and 10 min, and counting the amount of bound hot ligand. To test the effect of EtOH on the dissociation rate, 200 mM EtOH was added instead of cold Ro15-4513 to prevent rebinding of the radioligand after dissociation. This experiment is representative for a total of three experiments that were performed.

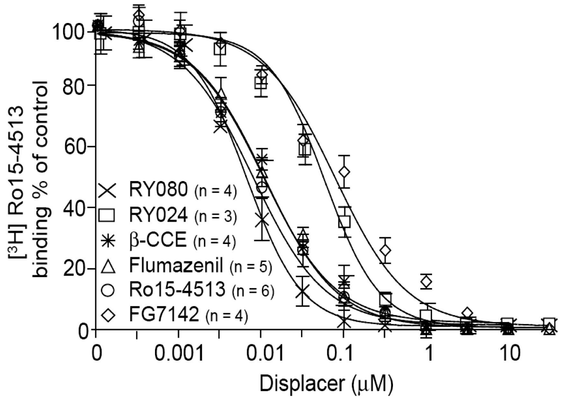

Fig. 4. Pharmacological characterization of the $[^3H]$Ro15-4513-binding site. Displacement of 10 nM $[^3H]$Ro15-4513 by the BZ antagonist flumazenil (Ro15-1788), the Ro15-4513 congeners RY080 and RY024, and the BZ site ligands and β-CCE on recombinant $\alpha_4\beta_3\delta$ receptors expressed in HEK cells. Very similar results were obtained displacing $[^3H]$Ro15-4513 with flumazenil, FG7142, and β-CCE from native immunopurified δ subunit-containing cow cerebellar $GABA_A$Rs (data not shown).

ies (29), ≈20% of the $[^3H]$Ro15-4513 binding to cerebellar membranes was not blocked by 10 μM DZ (Fig. 2*b Inset*). Of this DZ-insensitive $[^3H]$Ro15-4513 binding, ≈30% was dose-dependently inhibited by EtOH ($IC_{50} \approx 7$ mM), with a maximum inhibition at 100 mM EtOH (Fig. 2*b*). Given that DZ-IS binding of $[^3H]$Ro15-4513 in the cerebellum is ≈20% of total binding, EtOH-displaceable Ro15-4513 binding is ≈6% of total cerebellar Ro15-4513 binding. This percentage is consistent with the δ Ab-precipitated fraction, enriched in $\alpha_6\beta\delta$ receptors and in the same range as the fraction of $\alpha_6\beta_x\delta$ receptors (≈11%) determined by biochemical methods in rat cerebellum (30). EtOH did not inhibit the $[^3H]$Ro15-4513 binding to classical DZ-sensitive sites under the same conditions (data not shown).

Ro15-4513 Is a Competitive Alcohol Antagonist. The complete and dose-dependent displacement of $[^3H]$Ro15-4513 by EtOH on recombinant $\alpha_4\beta_3\delta$ receptors (Fig. 2*a*) suggests the possibility of a competitive antagonism between EtOH and Ro15-4513. To evaluate and distinguish competitive (direct) from allosteric (indirect) interaction further, we performed $[^3H]$Ro15-4513 saturation-binding experiments in the continuous presence of nonsaturating EtOH concentrations. Receptor occupancy of ligands (in our case, $[^3H]$Ro15-4513) decreases in a predictable way in the presence of a presumed competitive antagonist (EtOH). The inclusion of 10, 30, or 50 mM EtOH in $[^3H]$Ro15-4513 saturation-binding experiments led to a dose-dependent parallel shift of the $[^3H]$Ro15-4513 binding curve to the right (Fig. 3). The simultaneous least-square fit using a combined Hill/Schild–Gaddum equation (with K_d = 7.5 nM for Ro15-4513) resulted in a Hill coefficient of 1.1 and a K_d = 8.1 mM for EtOH (colored curves in Fig. 3). That the prediction for competitive antagonism overlaps with our experimental data provides additional evidence that alcohol and Ro15-4513 are likely to occupy overlapping binding sites.

The decrease in receptor occupancy in the presence of competitive antagonists, as described by the Schild–Gaddum equation, is due to a reduction in the apparent association rate of ligands (binding sites occupied by the competitive ligand are not available for binding), without changes in the dissociation rate (i.e., the residence time of ligands in their binding sites). Therefore, we decided to determine the dissociation rate of $[^3H]$Ro15-4513 from recombinant $\alpha_4\beta_3\delta$ receptors by measuring unbinding after the addition of excess cold (1 μM) Ro15-4513 or a high concentration of EtOH (200 mM) to a receptor preparation equilibrated with 5 nM $[^3H]$Ro15-4513. Excess (1 μM) Ro15-4513 as well as 200 mM EtOH led to essentially identical $[^3H]$Ro15-4513 ligand dissociation rates, showing that EtOH does not decrease the residence time of $[^3H]$Ro15-4513 in its receptor (Fig. 3 *Inset*). This result suggests that EtOH, even at 200 mM, does not allosterically alter the binding pocket.

Ro15-4513 Binding to $\alpha_4\beta_3\delta$ $GABA_A$Rs Is Inhibited by Ligands That Prevent the Behavioral Alcohol Antagonism of Ro15-4513. To further characterize the $[^3H]$Ro15-4513 binding site on the δ subunit receptors, we performed competition binding experiments on recombinant ($\alpha_4\beta_3\delta$) and native (cow cerebellum) immunopurified δ subunit-containing receptors with BZ site ligands that have been shown to reverse the behavioral alcohol antagonism of Ro15-4513 [flumazenil, β-CCE, and *N*-methyl-β-carboline-3-carboxamide (FG7142)] (22, 31) and the imidazo-BZs (Ro15-4513/flumazenil structural analogs) RY024 and RY080. RY024 has been described as a behavioral alcohol antagonist (32, 33). We show that like "cold" Ro15-4513, flumazenil, RY024, and RY080, and the β-carbolines β-CCE and FG7142, displaced $[^3H]$Ro15-4513 from its binding site on $\alpha_4\beta_3\delta$ receptors (Fig. 4). Therefore, ligands that block EtOH enhancement of $\alpha_{4/6}\beta_3\delta$ $GABA_A$R currents, such as Ro15-4513, or that reverse the alcohol-antagonist activity of Ro15-4513 (flumazenil and β-CCE) (21, 29, 45), are also able to displace $[^3H]$Ro15-4513 binding. However, most other BZ site ligands [including all of the tested classical BZ agonists (DZ, flurazepam, flunitrazepam, and midazolam), the β-carboline DMCM, and the BZ site ligands zolpidem and zopiclone] that are known to bind with high affinity to the classical BZ sites in γ_2 subunit-containing recep-

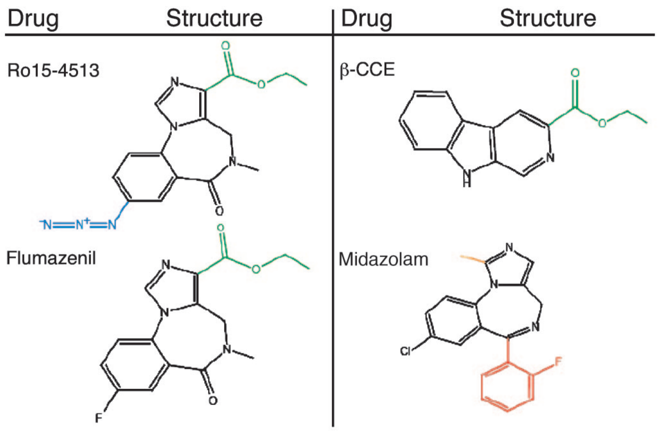

Fig. 5. Structure-affinity and activity relationship of 14 selected BZ site ligands. The structure of representative BZ site ligands is shown. The blue moiety differs between the alcohol/BZ antagonist Ro15-4513 and the general BZ (but not alcohol) antagonist flumazenil at the C7 position of the BZ ring structure. The carboxyethyl ester (position 3′ in imidazo-BZs) that is present in all four compounds that show affinities ≤10 nM is shown in green. The methyl group in the inactive compound midazolam that may cause steric hindrance is shown in orange. The pendant phenyl group (present in all classical BZ agonists but lacking in the high-affinity compounds Ro15-4513 and flumazenil) at a position that may prevent binding of classical BZs to the BZ/EtOH site on δ receptors is shown in red.

tors do not displace [^{3}H]Ro15-4513 from this binding site on δ receptors at reasonable concentrations (Fig. 5 and Table 1). This observation is consistent with the notion that these compounds will not prevent the alcohol-antagonist activity of Ro15-4513. The $GABA_AR$ channel antagonist picrotoxinin (100 μM) did not inhibit binding.

Flumazenil, Ro15-4513, and RY080 all differ only at the moiety at the C7 position (blue in Fig. 5; fluorine in flumazenil, an azido group in Ro15-4513, and an acetylene group in RY080). The less potent compound RY024 is identical to RY80 except that it contains the carboxy-*t*-butyl ester instead of the carboxyethyl ester moiety. Also, the only difference between FG7142 and β-CCE is that the lower-affinity compound FG7142 carries a carboxymethyl amide instead of a carboxyethyl ester. Therefore, it is likely that the carboxyethyl ester moiety (green in Fig. 5), which is present in all compounds with affinities of $K_d \leq 10$ nM (Table 1), is important for high-affinity binding to the alcohol/BZ site in $\alpha_{4/6}\beta_3\delta$ receptors. The lack of affinity of classical BZ agonists and DMCM could be due to the absence of the carboxyethyl ester group at the appropriate location. Also, chemical moieties in these inactive compounds (e.g., the pendant phenyl group in classical BZs; red in Fig. 5) may lead to steric hindrance.

Table 1. Activity of 13 selected BZ site ligands on the [^{3}H]Ro15-4513 binding site on $\alpha 4\beta 3\delta$ $GABA_A$Rs

Drug	K_i, nM
Ro15-4513	K_d 7.5
RY080	6.5
Flumazenil	8.3
β-CCE	10.4
FG7142	78.8
RY024	111.4
DMCM	>1,000
Bretazenil	≫1,000
Midazolam	≫1,000
Diazepam	≫1,000
Flunitrazepam	≫1,000
Flurazepam	≫1,000
Zopiclone	>1,000
Zolpidem	≫1,000

K_i values were determined based on the ability to displace [^{3}H]Ro15-4513 (5 nM) from recombinant $\alpha 4\beta 3\delta$ receptors harvested from transiently transfected HEK293 cells. The concentrations resulting in half-maximal inhibition of specific [^{3}H]Ro15-4513 binding were converted to K_i values by using the Cheng-Prusoff relationship (44) and the K_d value for [^{3}H]Ro15-4513 of 7.5 nM. K_i values above 1,000 nM are grouped into two categories: less than 10% inhibition (≫1,000) and between 10% and 50% inhibition at 1,000 nM (>1,000).

Discussion

We show that native immunopurified and recombinantly expressed δ subunit-containing receptors bind the imidazo-BZ Ro15-4513 (and flumazenil) with high affinity in binding assays, and that Ro15-4513 binding is abolished not only by the β-carboline BZ-site ligands β-CCE and FG7142 but also by low concentrations of EtOH. It is a widely held notion that the BZ ligand-binding site is limited to the α–γ_2 interface, and δ subunit-containing receptors are insensitive to all BZ site ligands, based on reports that agonists like DZ and flunitrazepam did not enhance expressed δ-containing receptor currents (18, 34). Therefore, the discovery that certain BZ-site ligands can bind with high affinity to δ subunit-containing receptors is surprising.

Additional BZ-binding sites, such as the Ro15-4513/EtOH site on $\alpha_{4/6}\beta_3\delta$ receptors described here, might contribute to the diverse pharmacological properties of clinically used BZ site ligands. This BZ/EtOH site on $\alpha_{4/6}\beta_3\delta$ receptors was difficult to detect because these receptors are of relatively low abundance, and (like the BZ sites on $\alpha_{4/6}\beta\gamma_2$ $GABA_A$Rs) they are essentially insensitive to classical BZ site agonists (e.g., DZ, flunitrazepam, midazolam, and flurazepam). Also, binding of the high-affinity BZ ligand Ro15-4513 to these $GABA_A$R subtypes is functionally silent (18) and, therefore, usually remains undetected in functional assays.

Our data are consistent with a competitive model of interaction between EtOH and Ro15-4513, suggesting that EtOH and Ro15-4513 occupy overlapping binding sites. In support of this concept, we showed that increasing concentrations of EtOH led to a complete inhibition of [^{3}H]Ro15-4513 binding to recombinant δ subunit-containing receptors, whereas binding of [^{3}H]Ro15-4513 to γ_2 subunit-containing receptors was not inhibited by EtOH. We also showed that, as expected from a simple competition for an overlapping binding site, the apparent affinity for Ro15-4513 was decreased in the presence of EtOH. Additional support for a competitive model comes from analysis of the unbinding reaction of [^{3}H]Ro15-4513. As expected for a competitive antagonism, excess EtOH (like excess cold Ro15-4513) led to virtually identical unbinding rates.

Structurally, Ro15-4513 is identical with the clinically used general BZ antagonist flumazenil, except that flumazenil carries a fluorine atom at position C7 of the BZ ring, whereas Ro15-4513 carries the larger azido group (Fig. 5). Given this structural similarity with flumazenil, it is not surprising that Ro15-4513 also binds with high affinity to the classical BZ sites and antagonizes the actions of classical BZ agonists (35), and that flumazenil (which by itself does not have alcohol-antagonist activity) can overcome the behavioral alcohol-antagonistic activity of Ro15-4513 (3, 22). Our results suggest that flumazenil, as well as β-CCE and FG7142, counteract the behavioral alcohol antagonism of Ro15-4513, because they displace Ro15-4513 from its binding site on δ subunit-containing receptors.

Also, that (*i*) Ro15-4513 and flumazenil differ only at the C7 position of the BZ ring structure, (*ii*) Ro15-4513, but not flumazenil, shows alcohol-antagonist properties, and (*iii*)

[^{3}H]flumazenil binding is not displaced by EtOH (≤300 mM) suggest that the C7 azido group is critical for alcohol antagonism. Therefore, we propose a model in which the azido group (which is approximately the same size as alcohol) occupies the EtOH-binding pocket in the Ro15-4513-bound receptor and thus prevents EtOH binding to these types of $GABA_AR$s. Consistent with this view, there have been recent reports of *in vivo* alcohol-antagonist actions of the Ro15-4513/flumazenil derivative RY024; RY024 and the related molecule RY080 have an acetylene group (instead of an azido group) at the C7 position of the BZ ring (32). We found that these chemical analogs of Ro15-4513 (RY024 and RY080) are potent inhibitors of [^{3}H]Ro15-4513 binding to this alcohol/BZ site. This inhibition suggests that the reported behavioral alcohol antagonism of RY024 may be due to reversal of alcohol actions on unique EtOH/BZ-sites on $GABA_AR$s.

Photolabeling, site-directed mutagenesis, cysteine-accessibility studies, and BZ pharmacophore models derived from those data lead to the consensus that side-chain residues at the C7 position of the BZ ring are close to the critical histidine residue (α_1-H102, bovine numbering) that determines sensitivity of $\alpha_{1,2,3,5}$-containing receptors to classical BZ agonists in $\alpha\beta\gamma_2$ receptors (36–40). The selective labeling, by the photoreactive C7 azido group of Ro15-4513, of tyrosine α_1-Y210 (bovine numbering) (37), which is in close proximity to α_1-H102 in structural homology models (37, 38, 41), is probably due to a higher photoreactivity of tyrosine residues (37).

The residue homologous to α_1-H102 in α_6 is polymorphic in rats and can be α_6-R100 or α_6-Q100. We showed that the α_6-100Q polymorphism, which has been selected for during breeding of alcohol hypersensitive (alcohol-nontolerant) rats (16), leads to a dramatically increased alcohol sensitivity of α_6-100Q$\beta_3\delta$ $GABA_AR$ *in vitro* and *in vivo* (12). Based on our findings described here, it is tempting to speculate that the reason for the increased alcohol sensitivity of α_6-100Q$\beta_3\delta$ receptors (and α_6-100Q/Q rats) is that the α_6-100 residue is very close to, and might even directly line, the EtOH-binding site.

The data presented here strongly support the view that the alcohol-antagonist action of Ro15-4513 is largely due to specific actions on alcohol-sensitive subtypes (like $\alpha_{4/6}\beta_3\delta$) of $GABA_AR$s. It remains to be determined whether the partial inhibition (inverse agonist action) of Ro15-4513 on some $GABA_AR$ subtypes contributes to this alcohol antagonism. However, the fact that other inverse agonists (e.g., FG7142 and β-CCE) are not alcohol antagonists (22, 24, 42) suggests that such a contribution from action on other $GABA_AR$ subtypes is only minor. The inverse agonists FG7142 and β-CCE (like flumazenil) reverse the behavioral alcohol antagonism of Ro15-4513 (22, 31) and displace Ro15-4513 from the EtOH/Ro15-4513 site on $\alpha_{4/6}\beta_3\delta$ receptors. Although the relative contributions of individual GABA and non-GABA receptors to the various aspects of EtOH intoxication, as well as whether there are other unrecognized subtypes of $GABA_AR$s that are also sensitive to EtOH/Ro15-4513 that may contribute to Ro15-4513-sensitive behavioral EtOH effects, remain to be determined, the results presented here strongly support the view that EtOH/Ro15-4513-sensitive $GABA_AR$s are important mediators of alcohol actions at blood-alcohol concentrations that are experienced during low and moderate alcohol consumption. Also, the competitive nature of the EtOH–Ro15-4513 interaction on δ subunit-containing $GABA_AR$ might allow us to determine where in the receptors the EtOH-binding site is located. The displacement of Ro15-4513 from $\alpha_{4/6}\beta_3\delta$ receptors provides a test-tube assay to screen for ligands that can bind to, or very close to, the EtOH-binding site that mediates behaviorally relevant low dose EtOH effects. This binding assay allows further structure–function analysis of the pharmacological potency and efficacy of compounds that are active at the "alcohol receptor." Ligands that are active at the alcohol/BZ binding site on δ subunit-containing $GABA_AR$ receptors may yield therapeutics for problems that are related to low dose EtOH sensitivity, including anxiety, insomnia, and alcoholism.

Methods

Radiolabeled [^{3}H]Ro15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo(1,5-*a*)BZ-3-carboxylate (33.3 Ci/mmol; 1 Ci = 37 GBq) was purchased from PerkinElmer) and was supplied dissolved in EtOH. To change the solvent from EtOH to DMSO, the [^{3}H]Ro15-4513 EtOH solution was dried in a vacuum concentrator and redissolved in DMSO. Unlike EtOH, DMSO at final concentrations (<1%) did not change [^{3}H]Ro15-4513 binding to cerebellar δ-IP receptors (data not shown).

Ro15-4513, flumazenil (ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]BZ-3-carboxylate), DZ, and flunitrazepam were obtained from Hoffman–LaRoche; DMCM was a gift from Ferrosan (Copenhagen); and FG7142 and β-CCE were provided by Schering. EtOH, GABA, and bicuculline were purchased from Sigma. Compounds were dissolved in DMSO as a 10 mM stock solution and used at the indicated concentrations. Protein concentration was determined with the BCA protein assay kit (Pierce) with BSA as standard.

Membrane Preparation. Bovine cerebellum was obtained from a local supplier and stored frozen at −70°C. Tissue was thawed and homogenized by sonication in 10 volumes of homogenization buffer [50 mM Tris·HCl, pH 8.0/50 mM KCl/1 mM EDTA/0.32 M sucrose/0.5 mM DTT/0.01% bacitracin supplemented with either protease inhibitors (2 mM benzamidine/0.1 mM benzethonium chloride/0.3 mM PMSF) or a protease inhibitor mixture (Complete Mini, Roche Diagnostics)] and centrifuged (550 × *g*) for 10 min at 4°C to pellet nuclei and cells. The supernatant fraction was collected by three sonication–centrifugation cycles (in homogenization buffer without sucrose). Membrane pellets were collected at 35,000 × *g* for 1 h at 4°C and used for ligand binding, or they were stored frozen at −20°C.

Recombinant Cell Expression. HEK 293T cells were transfected with rat cDNAs under the control of a CMV promoter ($\alpha/\beta/\delta$ or γ_2, 1:1:2) as described in ref. 43, and cells were harvested at 60–100 h after transfection. Membranes from these HEK 293T pellets were homogenized by sonication in 10 volumes of assay buffer (100 mM KCl/10 mM KH_2PO_4/K_2HPO_4, pH 7.5, at 4°C) with a protease inhibitor mixture (Complete Mini) and subjected to three centrifugation–resuspension cycles before being used for ligand binding assays.

[^{3}H]Ro15-4513 Ligand Binding Assay. Membranes (or IP receptors bound to protein G–agarose beads) were resuspended in assay buffer (50 mM Tris·HCl, pH 8.0/1 M KCl/1 mM EDTA/0.5 mM DTT/2 mM benzamidine/0.01% bacitracin/0.3 mM PMSF/10 μg/ml trypsin inhibitor) by sonication. Resuspended membranes were incubated (in a volume of 0.5 ml) for 60 min on ice in the presence of [^{3}H]Ro15-4513 (33.3 Ci/mmol, PerkinElmer) and various concentrations of competing ligands. Membranes (10–40 μg of protein per filter) were collected by rapid filtration on GF/B filters (Whatman). After three washing steps with 10 ml of assay buffer, the filter-retained radioactivity was counted in a LS3800 liquid scintillation counter (Beckman). Nonspecific binding was determined in the presence of 10 μM Ro15-4513, and DZ-IS binding was determined in the presence of 10 μM DZ. Data for binding curves were fitted by using a nonlinear least-squares method with the following equations: $B(c) = B_{max}/(1 + (K_d/c)^n)$, for binding curves, and $B(c) = B_{max}/(1 + (c/IC_{50})^n)$, where c is the concentration of ligand, B is binding, B_{max} is maximal binding, K_d is the dissociation constant, n is the Hill coefficient, and IC_{50} is the half-maximal inhibitory concentration. These calculations were performed by using PRISM (GraphPad, San Diego) software. All error bars in the figures indicate SD.

Immunoprecipitation (IP). IPs were performed from membrane preparations solubilized in assay buffer (as described above), supplemented with 8 mM of the nonionic detergent nonaoxyethylene dodecyl ether (C12E9), by using rabbit $GABA_AR$ δ subunit-specific Ab (30) and the protein G IP50 IP kit (Sigma). Briefly, 100 μl (≈1 μg/μl) of protein extracts were incubated in 600 μl of IP buffer plus 60 μl of 0.5 M NaCl with Ab (at appropriate dilutions) overnight at 4°C. After the addition of 30 μl of protein G–agarose, the tubes were incubated for an additional 2 h with mixing. The beads were washed five times with cold IP buffer, and binding assays were performed on Ab-bound receptors (30).

We thank Dr. James Cook (University of Wisconsin, Milwaukee) for kindly providing RY024 and RY080 compounds, Dr. Mogens Nielsen (Danish University of Pharmaceutical Sciences, Copenhagen) for critical comments on the manuscript, and Dr. Cameron Gundersen (University of California, Los Angeles) for sharing equipment. This work was supported by National Institutes of Health Predoctoral Fellowship AA015460 (to H.J.H.), an Alcoholic Beverage Medical Research Foundation grant (to M.W.), National Institutes of Health Grants NS35985 and AA07680, and funds provided by the State of California for medical research on alcohol and substance abuse (to R.W.O.). P.C. was supported by a scholarship from the Commission on Higher Education Staff Development Project of Thailand. W.S. was supported by Austrian Science Fund Project P15165.

1. Siggins, G. R., Roberto, M. & Nie, Z. (2005) *Pharmacol. Ther.* **107,** 80–98.
2. Criswell, H. E. & Breese, G. R. (2005) *Neuropsychopharmacology* **30,** 1407–1425.
3. Ticku, M. K. & Kulkarni, S. K. (1988) *Pharmacol. Biochem. Behav.* **30,** 501–510.
4. Aguayo, L. G., Peoples, R. W., Yeh, H. H. & Yevenes, G. E. (2002) *Curr. Top. Med. Chem.* **2,** 869–885.
5. Liljequist, S. & Engel, J. (1982) *Psychopharmacology* **78,** 71–75.
6. Mihic, S. J., Ye, Q., Wick, M. J., Koltchine, V. V., Krasowski, M. D., Finn, S. E., Mascia, M. P., Valenzuela, C. F., Hanson, K. K., Greenblatt, E. P., *et al*. (1997) *Nature* **389,** 385–389.
7. Wallner, M., Hanchar, H. J. & Olsen, R. W. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 15218–15223.
8. Hanchar, H. J., Wallner, M. & Olsen, R. W. (2004) *Life Sci.* **76,** 1–8.
9. Nusser, Z., Sieghart, W. & Somogyi, P. (1998) *J. Neurosci.* **18,** 1693–1703.
10. Wei, W., Zhang, N., Peng, Z., Houser, C. R. & Mody, I. (2003) *J. Neurosci.* **23,** 10650–10661.
11. Farrant, M. & Nusser, Z. (2005) *Nat. Rev. Neurosci.* **6,** 215–229.
12. Hanchar, H. J., Dodson, P. D., Olsen, R. W., Otis, T. S. & Wallner, M. (2005) *Nat. Neurosci.* **8,** 339–345.
13. Wei, W., Faria, L. C. & Mody, I. (2004) *J. Neurosci.* **24,** 8379–8382.
14. Carta, M., Mameli, M. & Valenzuela, C. F. (2004) *J. Neurosci.* **24,** 3746–3751.
15. Borghese, C. M., Storustovu, S. I., Ebert, B., Herd, M. B., Belelli, D., Lambert, J. J., Marshall, G., Wafford, K. A. & Harris, R. A. (2006) *J. Pharmacol. Exp. Ther.* **316,** 1360–1368.
16. Korpi, E. R., Kleingoor, C., Kettenmann, H. & Seeburg, P. H. (1993) *Nature* **361,** 356–359.
17. Sanna, A., Congeddu, E., Porcella, A., Saba, L., Pistis, M., Peis, M., Marchese, G., Ruiu, S., Lobina, C., Grayson, D. R., *et al*. (2003) *Brain Res.* **967,** 98–105.
18. Brown, N., Kerby, J., Bonnert, T. P., Whiting, P. J. & Wafford, K. A. (2002) *Br. J. Pharmacol.* **136,** 965–974.
19. Wieland, H. A., Luddens, H. & Seeburg, P. H. (1992) *J. Biol. Chem.* **267,** 1426–1429.
20. Benson, J. A., Low, K., Keist, R., Möhler, H. & Rudolph, U. (1998) *FEBS Lett.* **431,** 400–404.
21. Kolata, G. (1986) *Science* **234,** 1198–1199.
22. Suzdak, P. D., Glowa, J. R., Crawley, J. N., Schwartz, R. D., Skolnick, P. & Paul, S. M. (1986) *Science* **234,** 1243–1247.
23. Dar, M. S. (1995) *Pharmacol. Biochem. Behav.* **52,** 217–223.
24. Syapin, P. J., Jones, B. L., Kobayashi, L. S., Finn, D. A. & Alkana, R. L. (1990) *Brain. Res. Bull.* **24,** 705–709.
25. Miczek, K. A. & Weerts, E. M. (1987) *Science* **1987,** 1127–1128.
26. Sieghart, W., Eichinger, A., Richards, J. G. & Möhler, H. (1987) *J. Neurochem.* **48,** 46–52.
27. Möhler, H., Benke, D., Fritschy, J. M. & Benson, J. (2000) in *GABA in the Nervous System: The View at Fifty Years*, ed. Olsen, R. W. (Lippincott Williams & Wilkins, Philadelphia), pp. 97–112.
28. Luddens, H., Pritchett, D. B., Kohler, M., Killisch, I., Keinanen, K., Monyer, H., Sprengel, R. & Seeburg, P. H. (1990) *Nature* **346,** 648–651.
29. Turner, D. M., Sapp, D. W. & Olsen, R. W. (1991) *J. Pharmacol. Exp. Ther.* **257,** 1236–1242.
30. Pöltl, A., Hauer, B., Fuchs, K., Tretter, V. & Sieghart, W. (2003) *J. Neurochem.* **87,** 1444–1455.
31. Glowa, J. R., Crawley, J., Suzdak, P. D. & Paul, S. M. (1988) *Pharmacol. Biochem. Behav.* **31,** 767–772.
32. McKay, P. F., Foster, K. L., Mason, D., Cummings, R., Garcia, M., Williams, L. S., Grey, C., McCane, S., He, X., Cook, J. M. & June, H. L. (2004) *Psychopharmacology* **172,** 455–462.
33. Cook, J. B., Foster, K. L., Eiler, W. J., Jr., McKay, P. F., Woods, J., Jr., Harvey, S. C., Garcia, M., Grey, C., McCane, S., Mason, D., *et al*. (2005) *Alcohol Clin. Exp. Res.* **29,** 1390–1401.
34. Saxena, N. C. & Macdonald, R. L. (1996) *Mol. Pharmacol.* **49,** 567–579.
35. Bonetti, E. P., Burkard, W. P., Gabl, M., Hunkeler, W., Lorez, H. P., Martin, J. R., Möhler, H., Osterrieder, W., Pieri, L. & Polc, P. (1988) *Pharmacol. Biochem. Behav.* **31,** 733–749.
36. Duncalfe, L. L., Carpenter, M. R., Smillie, L. B., Martin, I. L. & Dunn, S. M. (1996) *J. Biol. Chem.* **271,** 9209–9214.
37. Sawyer, G. W., Chiara, D. C., Olsen, R. W. & Cohen, J. B. (2002) *J. Biol. Chem.* **277,** 50036–50045.
38. Kucken, A. M., Teissere, J. A., Seffinga-Clark, J., Wagner, D. A. & Czajkowski, C. (2003) *Mol. Pharmacol.* **63,** 289–296.
39. Berezhnoy, D., Nyfeler, Y., Gonthier, A., Schwob, H., Goeldner, M. & Sigel, E. (2004) *J. Biol. Chem.* **279,** 3160–3168.
40. Smith, G. B. & Olsen, R. W. (2000) *Neuropharmacology* **39,** 55–64.
41. Ernst, M., Brauchart, D., Boresch, S. & Sieghart, W. (2003) *Neuroscience* **119,** 933–943.
42. Bonetti, E. P., Burkhard, W. P., Gabl, M. & Möhler, H. (1985) *Br. J. Pharmacol.* **86,** 463P.
43. Meera, P., Wallner, M., Song, M. & Toro, L. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 14066–14071.
44. Cheng, Y. & Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22,** 3099–3108.
45. Wallner, M., Hanchar, H. J. & Olsen, R. W. (2006) *Proc. Natl. Acad. Sci. USA* **103,** 8540–8545.