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Ethanol and the GABA Receptor Complex: Studies With the Partial Inverse Benzodiazepine Receptor Agonist Ro 15-4513

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GLOWA, J. R., J. N. CRAWLEY, P. D. SUZDAK AND S. M. PAUL. *Ethanol and the GABA receptor complex: Studies with the partial inverse benzodiazepine receptor agonist Ro 15-4513.* PHARMACOL BIOCHEM BEHAV 31(3) 767–772, 1988.—Ethanol potentiates GABA-receptor-medated Cl^- ion flux in vitro and, at similar concentrations, has anxiolytic and intoxicating properties in vivo. The imidazobenzodiazepine, Ro 15-4513 (ethyl-8-azido-5,6-dihydro-5-meth-6-oxo-4H-imidazo(1,5-a)(1,4)benzodiazepine-3-carboxylate), is a potent partial inverse agonist of the benzodiazepine/GABA receptor which can antagonize the in vitro actions of ethanol in potentiating GABA receptor-mediated Cl^- ion flux. Moreover, several of the behavioral effects of ethanol are also antagonized by Ro 15-4513, and these effects can be demonstrated in several paradigms at doses of Ro 15-4513 that do not produce opposite behavioral effects. In contrast, in our studies, other benzodiazepine receptor antagonist and inverse agonists, including Ro 15-1788, FG-7142, and β-CCE were not able to antagonize these biochemical or behavioral effects of ethanol at doses that were without intrinsic effects. However, both Ro 15-1788 and β-CCE blocked the antagonism of ethanol's effects by Ro 15-4513, suggesting a role for the GABA receptor complex in the actions of ethanol. These studies provide further evidence that GABAergic neurones may mediate at least some of the behavioral and biochemical actions of low-to-moderate doses of ethanol.

Ethanol GABA Ro 15-4513 Conflict Intoxication Schedule-controlled responding Rats Mice

LITTLE is known concerning the biochemical substrates and (or) the potential neuronal mechanisms by which the behavioral effects of ethanol are mediated. Knowledge of ethanol's mechanism(s) of action may lead to a better understanding of the tolerance and dependence commonly observed following repeated administration of ethanol in both laboratory animals and man. The fact that ethanol shares similar behavioral effects with benzodiazepines and barbiturates suggests the possibility that these drugs may share a common neuropharmacological mechanism. Behaviorally, all three types of drugs have anxiolytic-like, anticonvulsant and muscle-relaxant actions (5,25), and exhibit cross-tolerance with each other when substituted following repeated administration (3). It is well accepted that benzodiazepines and barbiturates decrease neuronal excitability by augmenting the actions of gamma-aminobutyric acid (GABA), the principle inhibitory neurotransmitter in brain, in regulating chloride (Cl^-) ion conductance (12). Both benzodiazepines and barbiturates bind with high affinity to specific recognition sites associated with the postsynaptic $GABA_A$ receptor complex to modulate the receptor in a manner highly correlated with their potencies in producing behavioral effects (19). Ethanol has also been shown by several investigators to enhance GABAergic neurotransmission (25). Recently, we have applied a biochemical method used to characterize the effects of benzodiazepine and barbiturates on GABA-mediated Cl-flux in vitro, to study the effects of ethanol at this receptor (23). Specifically, ethanol (and other short-chain alcohols) have been shown to potentiate GABA-receptor-mediated ^{36}Cl-flux in a subcellular preparation obtained from rat cerebral cortex. We have also used this preparation to study the effects of various ben-

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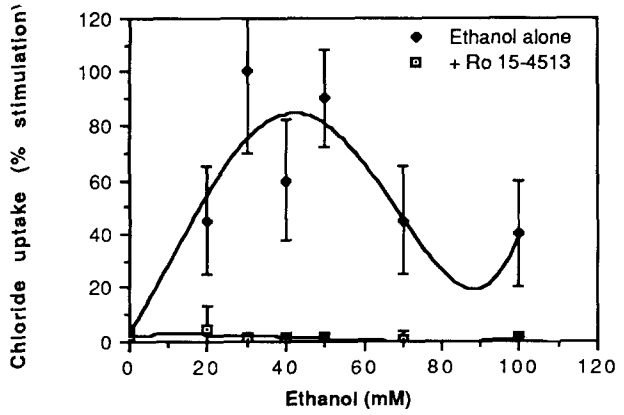

FIG. 1. Concentration-response curve for ethanol alone (closed diamonds) and presence (open squares) of Ro 15-4513 (100 nM) on the uptake of $^{36}Cl^-$ into cerebral cortical synaptoneurosomes. Data represent ±SEM [redrawn from (21)].

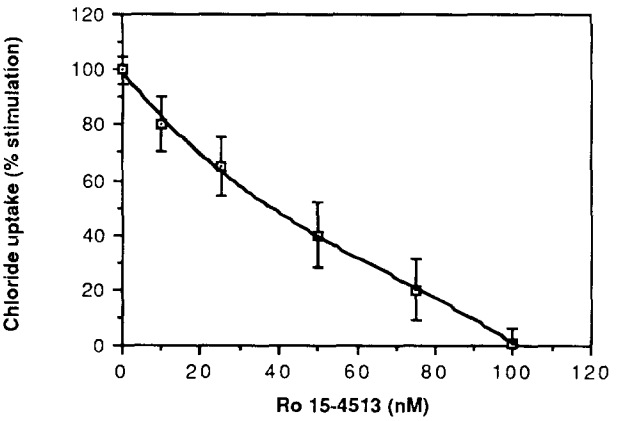

FIG. 2. The effect of Ro 15-4513 on ethanol stimulated ^{36}Cl-uptake in rat cortical synaptoneurosomes. Ro 15-4513 was added to synaptoneurosomes 5 min before the addition of ethanol (50 mM) and $^{36}Cl^-$. The data represent the mean±SEM of quadruplicate determinations [redrawn from (21)].

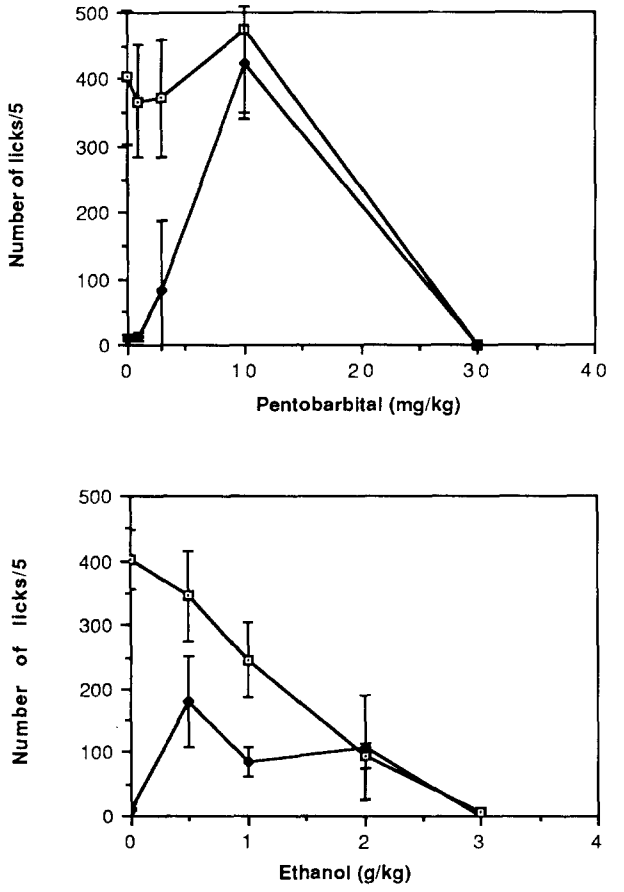

FIG. 3. The effects of ethanol and pentobarbital on suppressed (conflict) and nonsuppressed responding (licking) in rats. Experimentally naive Sprague-Dawley rats (150–200 g) were water-deprived 48 hr and placed in an operant chamber with an electrified water spout. Each 5th lick during a 10-min session produced a 0.5 mA shock (suppressed responding, closed triangles) or a 0 mA shock (nonsuppressed responding, open squares), for different rats. Pentobarbital sodium and ethanol (25% w/v) were given (IP) 10 min before sessions. Data represent mean±SEM for 6–8 rats.

zodiazepine receptor ligands (which previously have been shown to antagonize the behavioral effects of benzodiazepines) on ethanol stimulated ^{36}Cl-flux. During the course of studies we found that the imidazobenzodiazepine, Ro 15-4513, at low concentrations, selectively antagonized ethanol's actions at the GABA receptor complex. Moreover, we have shown that Ro 15-4513 also antagonizes the anticonflict and intoxicating effects of low to moderate doses of ethanol. Since Ro 15-4513 is a benzodiazepine receptor inverse agonist, we have attempted to delineate whether the antagonism of ethanol's behavioral actions by this compound is mediated through the benzodiazepine receptor and/or shared by other partial or full inverse agonists.

EFFECTS OF ETHANOL ON GABA RECEPTOR-MEDIATED Cl-FLUX

Using a subcellular brain preparation (the synaptoneurosomes) to study changes in Cl-ion flux induced by GABA and related agonists, we have examined the actions of ethanol and related short-chain alcohols. Briefly, rat cerebral cortex was gently homogenized, centrifuged, filtered, and resuspended to yield a preparation rich in pre- and postsynaptic membrane (synaptoneurosomes) (18). Ethanol, as well as other short-chain alcohols and barbiturates, either stimulate ^{36}Cl-uptake in to brain vesicles in a manner similar to that produced by GABA agonists such as muscimol or (at lower concentrations) potentiate muscimol-stimulated Cl-flux (23). These effects of ethanol occur at concentrations similar to those associated with ethanol's behavioral actions, such as sedation and intoxication. For example, ethanol stimulates uptake in the range of 20–80 mM, and, at subintoxicating concentrations of ethanol ≤20 mM, ethanol potentiates both muscimol and pentobarbital stimulated ^{36}Cl-uptake (21). The effect of ethanol on ^{36}Cl-uptake in this preparation is completely blocked by the GABA antagonists bicuculline and picrotoxin, but not by a number of other receptor antagonists (e.g., strychnine), or by several benzodiazepine receptor inverse agonists, including β-CCE, FG-7142, CGS 8216 and Ro 15-1788 (24). In subsequent experiments, the ability of the imidazobenzodiazepine, Ro 15-4513, to block ethanol-stimuated ^{36}Cl-uptake was studied. Ro 15-4513 potently antagonized ethanol-stimulated uptake,

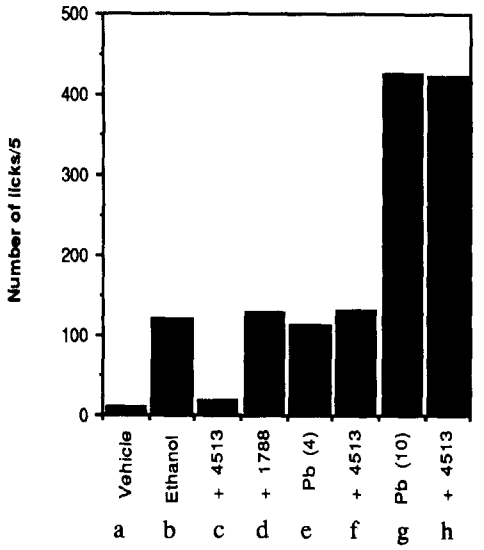

FIG. 4. The effects of Ro 15-4513 on ethanol- and pentobarbital-induced increases in conflict and their blockade by Ro 15-1788. Details as in Fig. 4. Conditions: (a) vehicle alone; (b) ethanol (1 g/kg); (c) ethanol (1 g/kg) + 3 mg/kg Ro 15-4513; (d) ethanol (1 g/kg) + 3 mg/kg Ro 15-4513 + 10 mg/kg Ro 15-1788; (e) 4 mg/kg pentobarbital; (f) 4 mg/kg pentobarbital + 3 mg/kg Ro 15-4513; (g) 10 mg/kg pentobarbital; (h) 10 mg/kg pentobarbital + 3 mg/kg Ro 15-4513.

with an ED_{50} of approximately 30–50 nM and complete antagonism was observed at approximately 100 nM, when tested against a maximally effective concentration of ethanol (50 mM) (Fig. 1). The ability of Ro 15-4513 to antagonize both high (Fig. 2) and low concentrations of ethanol was itself blocked by preincubation with the related imidazobenzodiazepine, Ro 15-1788, and the structurally unrelated benzodiazepine receptor antagonist CGS 8216. Taken together, these data suggest that relatively low concentrations of Ro 15-4513 can antagonize ethanol's action at the GABA receptor complex by binding to benzodiazepine recognition sites associated with the receptor.

EFFECTS ON CONFLICT RESPONDING IN RATS

Previous studies have shown that ethanol can increase punished responding in a variety of species (5, 8, 9). Similar effects are seen in a variety of agents possessing anxiolytic-like properties, such as the benzodiazepines (6), barbiturates (13) and other solvents (28), and, for the benzodiazepines, such effects have been highly correlated with anxiolytic efficacy in clinical settings (19). Our initial studies (24) assessed the effects of ethanol and pentobarbital on a modified version of punished responding (27). Ethanol (0.5–2.0 g/kg, 20% v/v, IP, 10 min before) increased shock-suppressed water-tube licking in naive, water-deprived rats (each fifth lick produced a 0.5 mA shock during 10-min sessions). The most consistent increase occurred at 1.0 g/kg, resulting in a 10-fold increase in the number of shocks produced, compared to control. Pentobarbital (IP, 10 min before) increased suppressed licking to an equal (4 mg/kg) or greater (10 mg/kg) extent than ethanol, depending upon the dose (Fig. 3). Under these conditions, Ro 15-4513 had no significant effect of suppressed or nonsuppressed licking at doses up to 3 mg/kg, and that dose, given 1 min before equipotent doses of ethanol and pentobarbital, effectively antagonized the rate-increasing actions of the former, but not the latter (Fig. 4). The ability of

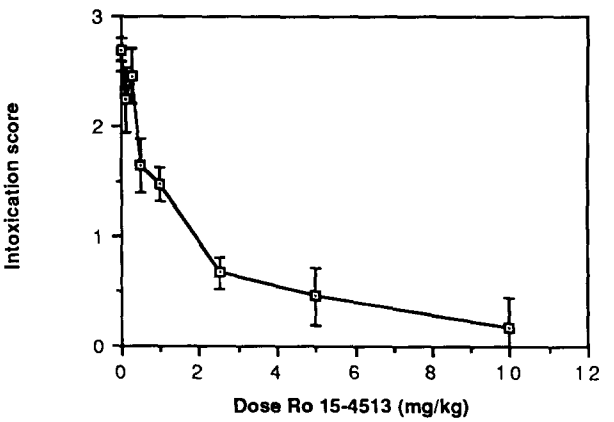

FIG. 5. Ro 15-4513 antagonism of ethanol-induced intoxication in the rat. Ro 15-4513 (0.1–10 mg/kg, IP) was administered 5 min before ethanol (2 g/kg, 20% v/v, IP); 8 min following ethanol rats were rated by an observer unaware of the treatment condition. Data represent the mean±SEM for at least 10 rats per dose [redrawn from (22)].

Ro 15-4513 to antagonize ethanol's effects was reversed by the central benzodiazepine receptor antagonist Ro 15-1788, further suggesting that the benzodiazepine/GABA receptor complex may be involved in the "antiethanol" actions of Ro 15-4513.

EFFECTS OF INTOXICATION IN RATS

The pharmacological profile of Ro 15-4513 in both blocking and reversing ethanol-induced intoxication in the rat has also been examined in several studies using the intoxication scale of Majchrowicz (14), with minor modifications, as a measure of motor dysfunction induced by moderate doses (2–3 g/kg) of ethanol. Each rat was rated by an observer, uninformed of the treatment conditions, on a scale of 0–4; 0=normal exploratory behavior, absence of easily observable signs of overt ethanol intoxication; 1=reduced muscle tone, moderate sedation, slowed locomotor activity, with some signs of motor incoordination and gait impairment; 2=pronounced sedation and motor incoordination and sluggish movements, slowed righting reflex, staggering gait, very little if any spontaneous movement, limbs extended away from the body; 3=very little or no recovery of righting reflex, profound sedation, no spontaneous motor activity, flaccid muscles, absence of pelvic and abdominal elevation; 4=complete loss of righting reflex, total immobility.

In the first series of experiments (20), male Sprague-Dawley rats (250–300 g) were given Ro 15-4513 (0.1–10 mg/kg, IP) or vehicle (4% Tween 80 in saline) 5 min before administration of ethanol (2 g/kg) and rated for intoxication 8 min after ethanol. Figure 5 shows that (at doses greater than 0.5 mg/kg) Ro 15-4513 antagonized ethanol-induced intoxication in a dose-dependent manner. Similar doses of Ro 15-4513 also antagonized intoxication produced by methyl (at 4.66 g/kg) and t-amyl (at 0.361 g/kg) alcohol, suggesting a similar mode of action for intoxication induced by other short-chain alcohols. The ability of Ro 15-4513 to antagonize ethanol-induced intoxication was blocked by the central benzodiazepine recpetor antagonists Ro 15-1788 and CGS 8216, suggesting the Ro 15-4513's antiintoxication effects were mediated through this receptor. However, surprisingly

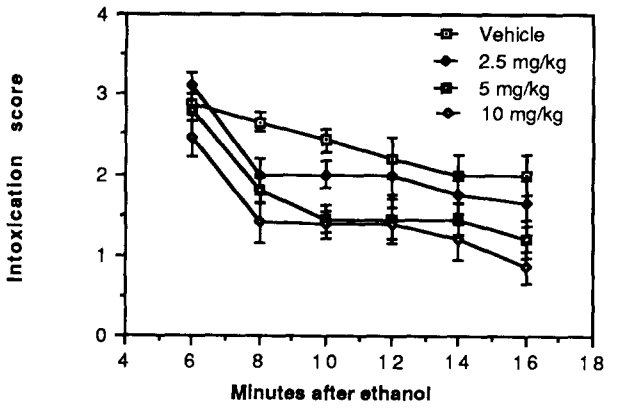

FIG. 6. Ro 15-4513 reversal of ethanol-induced intoxication in the rat. Six minutes following the administration of ethanol (2 g/kg, IP) animals were rated for intoxication. Animals then received either vehicle (open squares), 2.5 (closed diamonds), 5 (closed squares), or 10 (open diamonds) mg/kg Ro 15-4513 and were rated at two-minute intervals for an additional 10 min [redrawn from (22)].

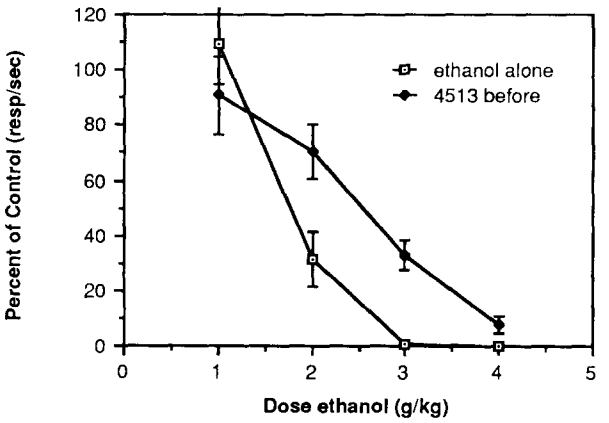

FIG. 7. The effects of ethanol alone (open squares) and in combination with Ro 15-4513 (10 mg/kg) given 1 min before ethanol, on FR 30 responding in adult male NIH mice. Effects were determined for approximately 30-min sessions, and are expressed as the mean effects for 6 mice, when responding was maintained under a FR 30 response schedule of milk presentation [redrawn from (7)].

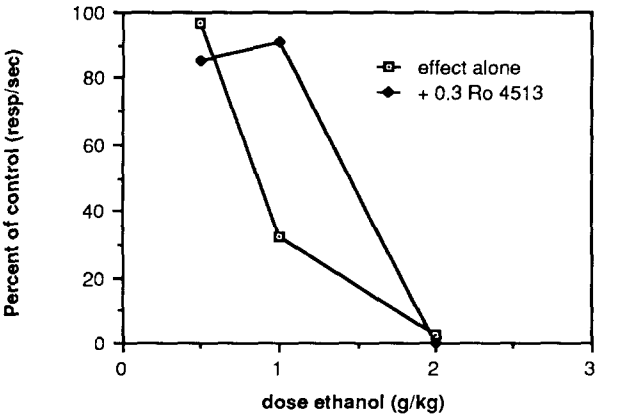

FIG. 8. Cumulative dose-effect function for ethanol alone (double determined, open squares), and in combination with Ro 15-4513 (0.3 mg/kg, closed triangles) given 5 min before the determination of a single cumulative dose-effect function. Effects are expressed as the mean effects for 10 mice, when responding was maintained under a FR 30 response LH 24-sec schedule of milk presentation [redrawn from (7), see also (21)].

the inverse agonists, β-CCE (10 mg/kg) and FG-7142 (5, 10 and 30 mg/kg) given 5 min before 2 g/kg ethanol, failed to alter ethanol-induced intoxication. Moreover, β-CCE, when administered immediately before Ro 15-4513, also blocked the ability of Ro 15-4513 to antagonize ethanol's actions.

In the second series of experiments Ro 15-4513 (2.5–10 mg/kg, or vehicle, IP) was given to rats 6 min after the administration of ethanol (2 g/kg) to investigate its ability to reverse ethanol-induced intoxication (21,22). Intoxication ratings were obtained immediately after Ro 15-4513 administration and every 2 min thereafter for an additional 10 min. Figure 6 shows that Ro 15-4513 (2.5 mg/kg) significantly reversed ethanol-induced intoxication at 8 min postethanol and the higher doses antagonized ethanol's effects for the 8–16 min period following ethanol administration. The inverse agonists FG-7142 or CGS 8216 failed to block ethanol intoxication, and, in addition, the antialcohol actions Ro 15-4513 were blocked by pretreatment with 10 mg/kg of Ro 15-1788 or CGS 8216, as in the earlier experiments.

EFFECTS ON FIXED RATIO RESPONDING IN MICE

In order to further assess the behavioral specificity of the blockade of ethanol's effects, the effects of ethanol, Ro 15-4513, and their combination, were studied on fixed-ratio (FR) performance in mice (4). NIH male mice had previously been trained to respond (poke their nose through a hole) under a FR 30-response schedule of food presentation (each 30th nose-poke produced access to 0.025 ml evaporated milk). Experimental sessions consisted of alternating periods in which responding could produce food (FR) and time-out (TO) periods during which food was not available, resulting in daily sessions of approximately 50 min. The effects of each drug were assessed after FR responding had stabilized at high rates (sessions were run 5 days per week). Ro 15-4513 was suspended in emulphor and diluted with sterile saline to produce a full range of doses given in a volume of 10 ml/kg body weight. Ethanol was given in a volume of saline necessary to produce the desired dose (approximately 0.06–0.48 ml).

In our initial experiments (7), the effects of several doses of ethanol (25% w/v, IP) were determined during entire daily sessions. Under these conditions, ethanol decreased FR responding at doses of 2–4 g/kg. The cumulative effects of Ro 15-4513 alone were then determined by incrementally increasing the dose during single experimental sessions (during the TO) until the entire dose-effect function was characterized. Consistent rate decreases occurred only at relatively high doses (30 mg/kg). The time course of Ro 15-4513 (30 mg/kg) was then determined during entire sessions, and the half-life was found to be approximately 15 min. Finally, the effects of a single dose of Ro 15-4513, given 1 min before single doses of ethanol, were studied. Figure 7 shows that Ro 15-4513 (10 mg/kg) antagonized the effects of intermediate doses of ethanol, shifting the dose-effect function approximately 1 g/kg to the right.

In subsequent experiments, the cumulative effects of various doses of ethanol (20% v/v, SC) and pentobarbital (SC) were assessed in the presence and absence of a low (0.3 mg/kg, SC) dose of Ro 15-4513. Both ethanol and pentobarbital decreased FR responding in a dose-dependent manner (see Fig. 8), but Ro 15-4513 only antagonized the effect of moderate doses of ethanol, and not those of pentobarbital.

DISCUSSION

The present results show that Ro 15-4513 can antagonize some of the biochemical and behavioral effects of ethanol. These data also suggest a primary role for GABA in these behavioral actions of ethanol. Ro 15-4513 has previously been shown to augment PTZ-induced seizures (2), and has well-described partial inverse agonist properties which may contribute to its ability to counteract some of the effects of ethanol (11). Previously it has been shown that several drugs which affect the chloride channel in a manner opposite to that of benzodiazepines can counteract the "antianxiety" effects of alcohol (8,10). However relatively high doses of these agents are required, and the possibility that these drugs are simply producing the opposite pharmacological action must be considered. Nevertheless, although Ro 15-4513 (0.5–10 mg/kg) could antagonize the behavioral effects of moderate doses of ethanol (2 g/kg), neither FG-7142 (5, 10, 30 mg/kg), nor β-CCE (10 mg/kg) were able to antagonize the behavioral actions of ethanol in the same conditions. Further, β-CCE actually antagonized Ro 15-4513's antiethanol effects (22); one might expect synergism if simply the inverse agonists properties of the latter were responsible for its antiethanol effects. Moreover, in our experiments with mice, the ability of Ro 15-4513 to antagonize the rate-decreasing effects of ethanol was associated with rate-decreasing effects of Ro 15-4513 itself. This appears inconsistent with the notion that opposite behavioral effects of each drug were responsible for the observed antagonism of ethanol's effects. These data further suggest that a simple inverse agonist action may be insufficient to explain the ability of Ro 15-4513 to antagonize the actions of ethanol in a number of behavioral paradigms. Whether other inverse agonists, however, will be found with similar "antialcohol" properties requires further investigation.

The inability of comparable doses of Ro 15-4513 to antagonize the behavioral effects of pentobarbital in some paradigms, the blockade of Ro 15-4513's antiethanol effect by Ro 15-1788 and CGS 8216, and the failure of several other inverse agonists to attenuate the biochemical and behavioral effects of ethanol, suggests a unique interaction of this drug with the GABA receptor complex. Previously, we have speculated that Ro 15-4513 may bind to a novel domain on the benzodiazepine/GABA receptor preventing a physicochemical change in receptor conformation induced by ethanol (24). Nevertheless, the precise mechanism(s) of action of Ro 15-4513 in blocking ethanol's actions remain unknown. These data, as well as that of others (1,15), suggests that Ro 15-4513 (and/or related compounds) will become important pharmacological tools in delineating the biochemical mechanisms associated with at least some of the behavioral effects of ethanol. In this regard, recent studies have demonstrated that Ro 15-4513 can block discriminative (16) as well as reinforcing (17) properties of ethanol. Such results suggest that the range of different behavioral effects of ethanol that may be related to augmentation of GABAergic transmission is sufficiently wide to approach a better understanding of factors related to the abuse potential of this drug by monitoring changes in GABA-related effects during repeated administration of ethanol.

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