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ORIGINAL INVESTIGATION

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# $GABA_B$ receptors play a role in the development of tolerance to ethanol in mice

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**Abstract** *Rationale:* There is evidence that drugs that improve or impair learning can facilitate or block ethanol tolerance, respectively. Since $GABA_B$ receptors have been shown to be involved in processes related to learning, it is possible that this system could play a role in the development of rapid tolerance to ethanol. *Objectives:* The aim of this study was to verify the influence of one $GABA_B$ agonist and two $GABA_B$ antagonists on tolerance to the effect of ethanol on motor coordination. *Methods:* Male Swiss mice were trained on a continuously accelerating rota-rod device. Animals were pretreated with the $GABA_B$ agonist (-)-baclofen (3, 5, or 7 mg $kg^{-1}$) or saline, 30 min before ethanol (1.75 g $kg^{-1}$), and were tested 5, 10, and 15 min later on the rota-rod. In another set of experiments, mice were pretreated with the $GABA_B$ antagonists CGP36742 (1, 3, 10, or 30 mg $kg^{-1}$) or CGP56433 (0.1, 0.3, 1.0, or 3.0 mg $kg^{-1}$), or saline, 30 min before the test under ethanol. Rapid tolerance was evaluated 24 h after the first ethanol injection, by injecting all animals with ethanol and retesting them on the rota-rod. *Results:* The results showed that (-)-baclofen (5 mg $kg^{-1}$) significantly (ANOVA + Tukey's test) blocked rapid tolerance, whereas CGP36742 (3 and 10 mg $kg^{-1}$) and CGP56433 (0.3, 1, and 3 mg $kg^{-1}$) facilitated rapid tolerance in a dose-dependent way. The blockade of rapid tolerance by (-)-baclofen was antagonized by previous administration of CGP36742 or CGP56433. *Conclusions:* The current results suggest that rapid tolerance to ethanol is subjected to inhibition by a GABAergic $GABA_B$ receptor-mediated system in the mouse.

**Keywords** Ethanol · $GABA_B$ · Rapid tolerance · CGP36742 · CGP56433 · (-)-Baclofen

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## Introduction

The development of tolerance to the effects of alcohol is considered an important element of alcohol dependence, since it favors the increase in alcoholic beverage consumption by reducing the aversive effect of alcohol with regard to its rewarding effects (Kalant 1996). Rapid development of tolerance to ethanol is seen when a second dose of this drug is given within 8–24 h after the initial dose of ethanol has been eliminated (Crabbe et al. 1979). In addition, rapid tolerance has been considered as a predictor of chronic tolerance (the form of tolerance that develops after days, weeks, or months of ethanol ingestion) because similar results were obtained with rapid and chronic paradigms involving ethanol tolerance or cross-tolerance between ethanol and other drugs (Khanna et al. 1991). Moreover, rapid tolerance seems to be predominantly functional and to involve processes of learning (Khanna et al. 1992). There is evidence that several drugs influencing learning and memory affect tolerance to ethanol. Drugs that impair learning, such as excitatory amino acid receptor antagonists and nitric oxide synthase inhibitors (Baron and Moerschbaecher 1996; Chapman et al. 1992), block tolerance to different ethanol effects (Barreto et al. 1998; Khanna et al. 1993, 1998; Morato and Khanna 1996). Conversely, agents that improve memory, such as D-cycloserine or tryptophan, facilitate tolerance (Khanna et al. 1994, 1995).

Although in recent years studies on the role of the GABAergic system in the development of tolerance have been intensified, efforts were directed towards the $GABA_A$ receptor (Hoffman et al. 1990; Karcz-Kubicha and Liljequist 1995; Tabakoff et al. 1996) and less is known about the interactions between alcohol and $GABA_B$ receptor-mediated mechanisms. The $GABA_B$ antagonist phaclofen has been shown to reverse the anticonvulsant effect of ethanol against picrotoxin (Mehta and Ticku 1990) and to increase the motor hyperexcitability observed during ethanol withdrawal in mice (Mead and Little 1995). Moreover, ethanol can induce a

$GABA_B$ receptor-dependent upregulation of central $GABA_A$-specific binding sites (Mizutani et al. 1993).

Davies et al. (1991) reported that $GABA_B$ receptors also have a modulatory function in long-term potentiation, a process considered to be an essential synaptic substrate for certain types of memory (Bliss and Collingridge 1993). In a study to verify the cognitive performance of mice, rats, and monkeys, Mondadori et al. (1993) showed that $GABA_B$ receptor blockers facilitate learning when these animals are submitted to passive avoidance, social learning, or color-space tests. Considering these pieces of evidence and the fact that tolerance is influenced by learning, the purpose of the present study was to investigate the role of $GABA_B$ receptors in the development of rapid tolerance to ethanol-induced motor impairment in mice. We used the selective $GABA_B$ receptor agonist (-)-baclofen (Hill and Bowery 1981; McNamara and Skelton 1996) and the selective $GABA_B$ receptor antagonists CGP36742 and CGP56433 (Mondadori et al. 1993; Pozza et al. 1999).

## Materials and methods

### Subjects

Adult male Swiss mice from the Universidade Federal de Santa Catarina's colony, 2–2.5 months of age and weighing 25–30 g, were used. The animals were bred in house at the university's animal house and transferred to our department's facilities at least 2 weeks prior to use, where they were housed in groups of 20 in plastic cages (42×34×17 cm), and maintained at 23±1°C under artificial illumination (lights on between 6 a.m. and 6 p.m.) with standard laboratory chow and tap water ad libitum. All experiments were conducted between 1 h 30 min and 5 h 30 min in order to minimize circadian influences, and all animals were naive to drug treatment and experience. All procedures were approved by our Institutional Ethics Committee and are in accordance with the National Institutes of Health Animal Care Guidelines.

### Apparatus

Motor impairment was measured on the rota-rod apparatus (Rotamex-V-EE/85) controlled by a computational system (Columbus Instruments Computer-Counter Interface, Columbus, Ohio, USA). Animals were trained under continuous acceleration (1 rpm/s) in 1-min sessions. Whenever an animal dropped off the rotating bar, it received a foot shock (0.1 mA for 2 s) and was then returned to its cage. The latency (in seconds) to fall off the rotating bar, was taken as the performance score. Animals that did not reach a stable baseline (i.e., remaining on the rotating bar for at least 20 s) in ten trials were discarded. The animals that presented performances between 20–40 s were selected for the experiment (average 27 s). The percentage of animals that usually attained the criterion was 90.9%. After the selection, experimental and control groups ($n$=10) were arranged according to their body weight and mean performance during the last training sessions. With this procedure, animals presented similar basal values in all groups. The baseline score was the score obtained by each animal before any treatment on one specific day (day 1 or day 2). The test score was the score obtained by each mouse at 5, 10, or 15 min after ethanol (or control) injections. The lowest test score obtained in each test session was used to calculate the maximum percent of motor impairment, according to:

$$\text{Maximum percent of motor impairment} = \frac{(\text{baseline score}) - (\text{test score})}{\text{baseline score}} \times 100 \quad (1)$$

### Drugs

Analytical grade ethanol was purchased from Merck Laboratory (Darmstadt, Germany) and was subsequently prepared by dilution in 0.9% NaCl (saline) to the concentration of 14% w/v. (-)-Baclofen, 3-aminopropyl-n-butyl-phosphinic acid (CGP36742) and [3-[1-(S)-[[3-(cyclohexylmethyl)-hydroxyphosphinyl]-2–(S)-hydroxypropyl]amino]ethyl]-benzoic acid (CGP56433) were supplied by Novartis (Basel, Switzerland) and were dissolved in saline. All drugs were freshly prepared and administered by the intraperitoneal (i.p.) route. Reagents for the determinations of blood ethanol levels were obtained from Sigma Chemicals (São Paulo, Brazil; Poklis and Mackell 1982). The dose range and pretreatment time of (-)-baclofen and $GABA_B$ receptor antagonists we used were based on the literature (McNamara and Skelton 1996; Steulet et al. 1996) and on our preliminary experiments.

### Procedure

In order to obtain rapid tolerance, trained mice were injected with 1.75 g $kg^{-1}$ ethanol or saline and were tested on the rota-rod at 5, 10, and 15 min after injections. Two hours after administration, each animal received an additional injection of saline or 1.25 g $kg^{-1}$ ethanol, according to the preceding treatment, in order to complete a total ethanol dose of 3 g $kg^{-1}$ (in the animals previously given 1.75 g $kg^{-1}$ of the drug). The use of two divided doses of ethanol on day 1 (i.e., 1.75+1.25 g $kg^{-1}$) was employed because previous experiments showed that a total dose of 3 g $kg^{-1}$ is required on day 1 to produce a reliable rapid tolerance on day 2. Since doses greater than 1.75 g $kg^{-1}$ may not invariably fall into the linear component of the dose-response curve, the use of this dose permits us to compare results over days (Barreto et al. 1998; Khanna et al. 1992). Mice were then returned to their home cages. Twenty-four hours later, all animals received 1.75 g $kg^{-1}$ ethanol and were tested at 5, 10, and 15 min on the rota-rod. In all experiments, data are presented as mean ± SEM of maximum percent motor impairment.

### Influence of (-)-baclofen on rapid tolerance to ethanol

This experiment was undertaken in order to verify whether a $GABA_B$ receptor agonist would block tolerance to ethanol. On day 1, three groups of 20 trained mice were pretreated with one of three doses of (-)-baclofen (3, 5, or 7 mg $kg^{-1}$), respectively, and another three groups received saline (control groups). After 30 min, each group was further divided into two groups that received ethanol (1.75 g $kg^{-1}$) or saline, respectively. Thus, four groups of ten animals for each dose of (-)-baclofen were formed. All animals were tested on the rota-rod at 5, 10, and 15 min after the last injection and then returned to their home cages. Two hours after administration, each animal received an additional injection of saline or 1.25 g $kg^{-1}$ ethanol, according to the preceding treatment, in order to complete 3 g $kg^{-1}$. Mice were then returned to their home cages until day 2, when the influence of the previous administration of (-)-baclofen on ethanol tolerance was measured according to the general procedure for acquisition of rapid tolerance (i.e., all animals were injected with 1.75 g $kg^{-1}$ ethanol and were tested on the rota-rod at 5, 10, and 15 min). A general scheme for the procedure followed in this and the next experiment is presented in Fig. 1.

### Influence of CGP36742 and of CGP56433 on rapid tolerance to ethanol

In order to verify whether the $GABA_B$ receptor antagonists CGP36742 and CGP56433 could facilitate tolerance, we used a total dose of 2.75 g $kg^{-1}$ (1.75+1 g $kg^{-1}$) ethanol which, unlike the total dose of 3 g $kg^{-1}$, does not induce rapid tolerance per se. In this experiment, four groups of 20 trained mice were pretreated

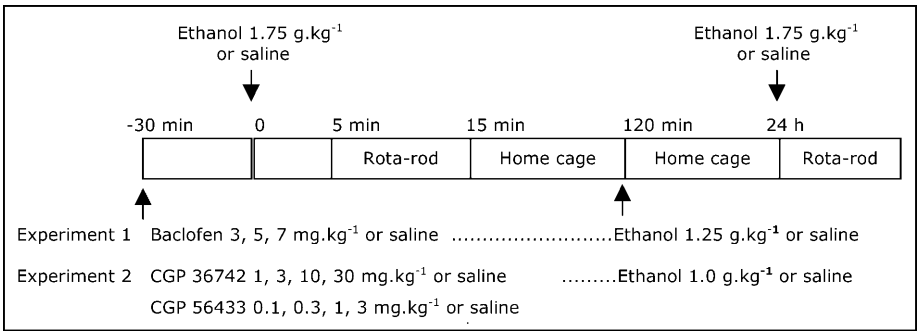


**Fig. 1** Schematic representation of experimental procedures used in experiments 1 and 2

with CGP36742 (1, 3, 10, or 30 mg kg$^{-1}$) and another four groups were treated with saline, respectively. An identical number of groups underwent a similar pretreatment protocol with CGP56433 (0.1, 0.3, 1.0, or 3.0 mg kg$^{-1}$) or saline. After 30 min, each group was further divided into two groups in order to receive ethanol (1.75 g kg$^{-1}$) or saline. Thus, four groups of ten animals for each dose of CGP36742 or CGP56433 were formed. The procedure employed in this experiment was then identical to that followed in experiment 1, except for the reduction in the second ethanol injection given (at 2 h on day 1) to complete the total dose of 2.75 g kg$^{-1}$ (1.0 instead of 1.25 g kg$^{-1}$; see Fig. 1).

### Influence of pretreatment with CGP36742 or CGP56433 on the blockade of rapid tolerance to ethanol by (-)-baclofen

This experiment was undertaken in order to verify whether the $GABA_B$ receptor antagonists could interfere in the blockade of rapid tolerance produced by (-)-baclofen. The protocol followed in this experiment was similar to that presented in the Fig. 1, except that the $GABA_B$ receptor antagonists (or their respective control vehicles) were administered 20 min before (-)-baclofen. Two groups of trained mice were pretreated with CGP36742 (3 or 10 mg kg$^{-1}$) and another two groups were treated with saline, respectively. After 20 min each group was divided into two groups that received (-)-baclofen (5 mg kg$^{-1}$) or saline. After an additional 30 min, each new group was further divided into two groups to receive ethanol (1.75 g kg$^{-1}$) or saline, respectively. Therefore, eight groups of animals were formed for each dose of CGP36742. All mice were tested on the rota-rod at 5, 10, and 15 min after the last injections. The animals were returned to their home cages. Two hours later, part of the animal groups received an additional dose of ethanol to complete 3 g kg$^{-1}$ and the remaining groups received saline, according to the treatment they received before the test. Mice were returned to their home cages. After 24 h, all animals received ethanol (1.75 g kg$^{-1}$) and were tested on the rota-rod (day 2).

A similar set of experiments was carried out following the same protocol except that one of three doses of the $GABA_B$ receptor antagonist CGP56433 were used (0.3, 1.0, or 3.0 mg kg$^{-1}$). Parallel control groups were run similarly to those used in the previous experiments.

### Blood ethanol assay

Groups of animals were pretreated with different doses of $GABA_B$ receptor agonist or antagonists 30 min before ethanol (1.75 g kg$^{-1}$). Blood samples were collected from animals by direct tail puncture 5 min after ethanol administration. Blood ethanol concentration was evaluated enzymatically based on ethanol's conversion to acetaldehyde by the action of alcohol dehydrogenase (Poklis and Mackell 1982).

### Data analysis

The Statistica for Windows 4.5 (Statsoft, Tulsa, Okla., USA) software was used to perform the statistical analysis. The difference between the baseline and maximal impairment scores provided the maximal percentage of motor impairment induced by ethanol. Data were analyzed using a multifactorial analysis of variance with pretreatments, treatment, and days as independent variables. Post hoc comparisons were performed using Tukey's test. Values of $P<0.05$ were considered significant. Figures were drawn using GraphPad Prism 1.03 (GraphPad Software, San Diego, Calif., USA).

## Results

### Influence of (-)-baclofen on the development of rapid tolerance to ethanol induced motor impairment

Figure 2A–C illustrates the results obtained with the injections of (-)-baclofen 30 min prior to alcohol treatment on day 1 on the motor coordination of mice tested 24 h later on the rota-rod under ethanol. A three-way ANOVA for repeated measures indicated significant differences for the treatment with ethanol [Fig. 2A: $F(1,36)=15.01$, $P<0.001$; Fig. 2B: $F(1,36)=125.80$, $P<0.0001$; Fig. 2C: $F(1,36)=26.75$, $P<0.0001$]. The post hoc comparisons showed significant differences between ethanol and control groups on day 1, demonstrating the acute effect of ethanol ($P<0.05$; Tukey's test). There was a significant effect of repeated factor (day 1 versus day 2) suggesting that the groups treated with ethanol on both days showed tolerance [Fig. 2A: $F(1,36)=9.27$, $P<0.01$; Fig. 2B: $F(1,36)=91.07$, $P<0.0001$; Fig. 2C: $F(1,36)=35.11$, $P<0.0001$]. Post hoc comparisons confirmed the development of tolerance to ethanol on day 2 in the groups pretreated with saline on day 1 and treated with ethanol on both days ($P<0.05$; Tukey's test). (-)-Baclofen 3 mg kg$^{-1}$ neither influenced the motor impairment of controls on day 1 nor the tolerance to ethanol on day 2. However, ANOVA did reveal a significant effect of pretreatment with (-)-baclofen at doses of 5 mg kg$^{-1}$ [$F(1,36)=8.33$, $P<0.01$] or 7 mg kg$^{-1}$ [$F(1,36)=49.45$, $P<0.0001$]. A significant pretreatment×treatment×day interaction was found for either 5 mg kg$^{-1}$ [$F(1,36)=11.73$, $P<0.01$] or 7 mg kg$^{-1}$ (-)-baclofen [$F(1,36)=74.57$, $P<0.0001$]. Post hoc analysis showed that (-)-baclofen 5 mg kg$^{-1}$ blocked the development of tolerance to ethanol without affecting the behavior of controls on day 1,

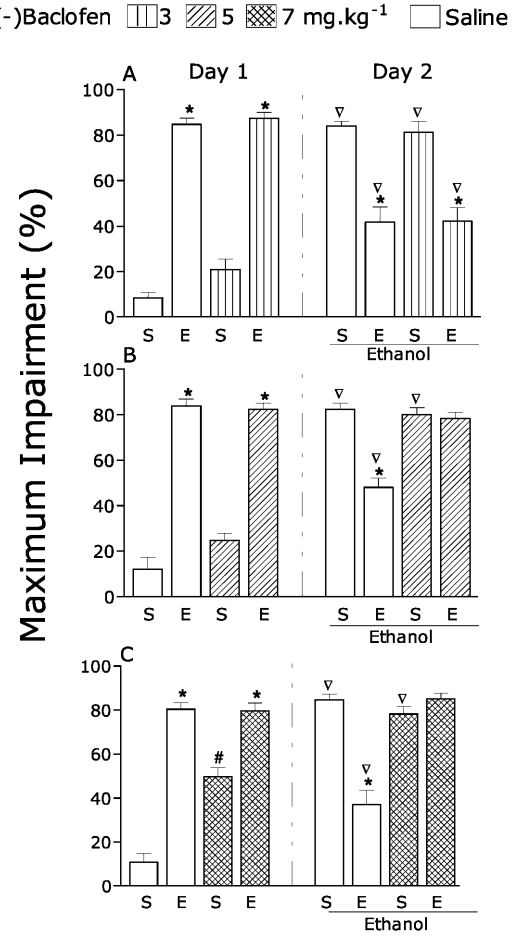


**Fig. 2A–C** Influence of (-)-baclofen on the development of rapid tolerance to ethanol-induced motor impairment. On day 1, control groups received saline and experimental groups received (-)-baclofen (3, 5, or 7 mg kg$^{-1}$; **A–C**, respectively) 30 min prior to saline (*S*) or ethanol (*E*; 1.75 g kg$^{-1}$), and were then tested on the rota-rod. On day 2, all groups were injected only with ethanol (1.75 g kg$^{-1}$) and tested on the rota-rod. Results shown are means ± SEM of ten animals per group. * $P$<0.05 compared to respective control on the same day, ▽ $P$<0.05 compared to performance of the same group on the previous day, # $P$<0.05 compared to performance of saline-pretreated saline-treated group on day 1 (Tukey's test)

whereas at 7 mg kg$^{-1}$ this drug also blocked the development of tolerance to ethanol, but produced a motor incoordinating effect per se on day 1. Taken together, these results suggest that pretreatment with the $GABA_B$ receptor agonist (-)-baclofen blocked the development of rapid tolerance to ethanol-induced motor impairment.

## Influence of CGP36742 and of CGP56433 on the development of rapid tolerance to ethanol-induced motor impairment

Figure 3A–D illustrates the influence of pretreatment with CGP36742 (1, 3, 10, or 30 mg kg$^{-1}$) prior to ethanol on day 1, on the development of rapid tolerance evaluated on day 2. A total dose of 2.75 g kg$^{-1}$ ethanol (1.75+1.00 g kg$^{-1}$, on day 1) failed to produce rapid tolerance per se, as evidenced by the similarity in performance on the rota-rod on day 2 (under the influence of ethanol 1.75 g kg$^{-1}$) of mice treated on day 1 with either ethanol or saline. Three-way ANOVA for repeated measures indicated a significant effect of the treatment with ethanol [Fig. 3A: $F(1,36)=182.30$, $P$<0.0001; Fig. 3B: $F(1,36)=193.55$, $P$<0.0001; Fig. 3C: $F(1,36)=64.90$, $P$<0.0001; Fig. 3D: $F(1,36)=262.63$, $P$<0.0001]. Post hoc comparisons showed significant differences between ethanol and control groups on day 1, indicating the acute effect of ethanol (Tukey's test). There was also a significant day effect [Fig. 3A: $F(1,36)=543.04$, $P$<0.0001; Fig. 3B: $F(1,36)=124.01$, $P$<0.0001; Fig. 3C: $F(1,36)=80.07$, $P$<0.0001; Fig. 3D: $F(1,36)=943.76$, $P$<0.0001], as well as a significant effect of pretreatment with CGP36742 at the doses of 3, 10, and 30 mg kg$^{-1}$ [Fig. 3B: $F(1,36)=19.16$, $P$<0.0001; Fig. 3C: $F(1,36)=21.61$, $P$<0.0001; Fig. 3D: $F(1,36)=80.86$, $P$<0.0001]. Moreover, ANOVA revealed significant pretreatment×treatment×day interactions [Fig. 3B: $F(1,36)=13.24$, $P$<0.001; Fig. 3C: $F(1,36)=10.23$, $P$<0.01; Fig. 3D: $F(1,36)=37.10$, $P$<0.0001]. Post hoc analysis showed that acute pretreatment with 30 mg kg$^{-1}$ CGP36742 significantly reduced the incoordinating effect of ethanol. Groups pretreated with CGP36742 (3 and 10 mg kg$^{-1}$) before ethanol on day 1 showed significant reductions of motor impairment on the 2nd day of the experiment. Post hoc analysis indicated the development of rapid tolerance for these groups compared to controls, suggesting that CGP36742 (3 and 10 mg kg$^{-1}$) facilitated the development of rapid tolerance to ethanol. The dose of 30 mg kg$^{-1}$ of CGP36742 produced a significant antagonism of the acute effect of ethanol, but did not facilitate the development of tolerance.

The effects of CGP56433 on ethanol tolerance are depicted in Fig. 4A–D. Confirming the observations collected in the previous set of experiments (Fig. 3), a total dose of 2.75 g kg$^{-1}$ ethanol on day 1 again did not produce rapid tolerance per se. A three-way ANOVA for repeated measures indicated significant effect of the treatment with ethanol [Fig. 4A: $F(1,36)=262.41$, $P$<0.0001; Fig. 4B: $F(1,36)=444.89$, $P$<0.0001; Fig. 4C: $F(1,36)=100.49$, $P$<0.0001; Fig. 4D: $F(1,36)=297.71$, $P$<0.0001]. Post hoc comparisons showed significant differences between ethanol and control groups on day 1, indicating the acute effect of ethanol (Tukey's test). The ANOVA also revealed a significant day effect [Fig. 4A: $F(1,36)=193.37$, $P$<0.0001; Fig. 4B: $F(1,36)=212.49$, $P$<0.0001; Fig. 4C: $F(1,36)=100.49$, $P$<0.0001; Fig. 4D: $F(1,36)=217.00$, $P$<0.0001]. Post hoc comparisons indicated significant differences between the groups treated with 0.3, 1, and 3 mg kg$^{-1}$ CGP56433 plus ethanol on day 1 and challenged with ethanol on day 2 and their respective controls (Fig. 4B–D, respectively) suggesting that CGP56433 (0.3, 1, and 3 mg kg$^{-1}$) facilitates the development of rapid tolerance to ethanol. ANOVA also revealed a significant effect of pretreatment with CGP56433 only with the doses of 0.3, 1, and 3 mg kg$^{-1}$

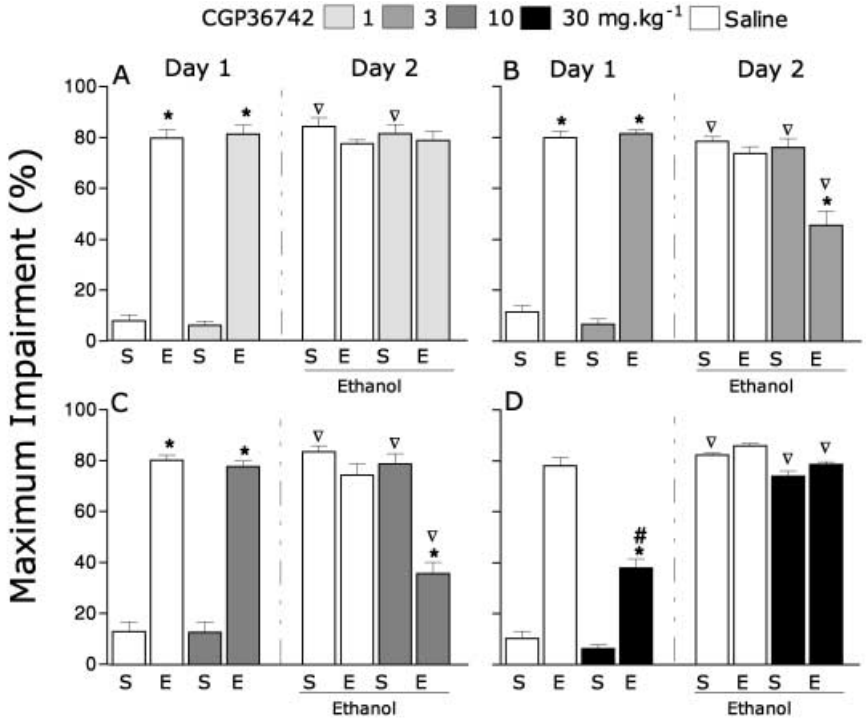


**Fig. 3A–D** Influence of CGP36742 on the development of rapid tolerance to ethanol-induced motor impairment. On day 1, control groups received saline (*S*) and experimental groups received CGP36742 (1, 3, 10, or 30 mg kg$^{-1}$; **A–D**, respectively) 30 min prior to saline or ethanol (*E*; 1.75 g kg$^{-1}$), and were then tested on the rota-rod. After 24 h, all groups were injected with ethanol (1.75 g kg$^{-1}$) and tested on the rota-rod (day 2). Results shown are means ± SEM of ten animals per group. * $P$<0.05 compared to respective control on the same day, ▽ $P$<0.05 compared to performance of the same group on the previous day, # $P$<0.05 compared to saline-pretreated ethanol-treated group on the same day (Tukey's test)

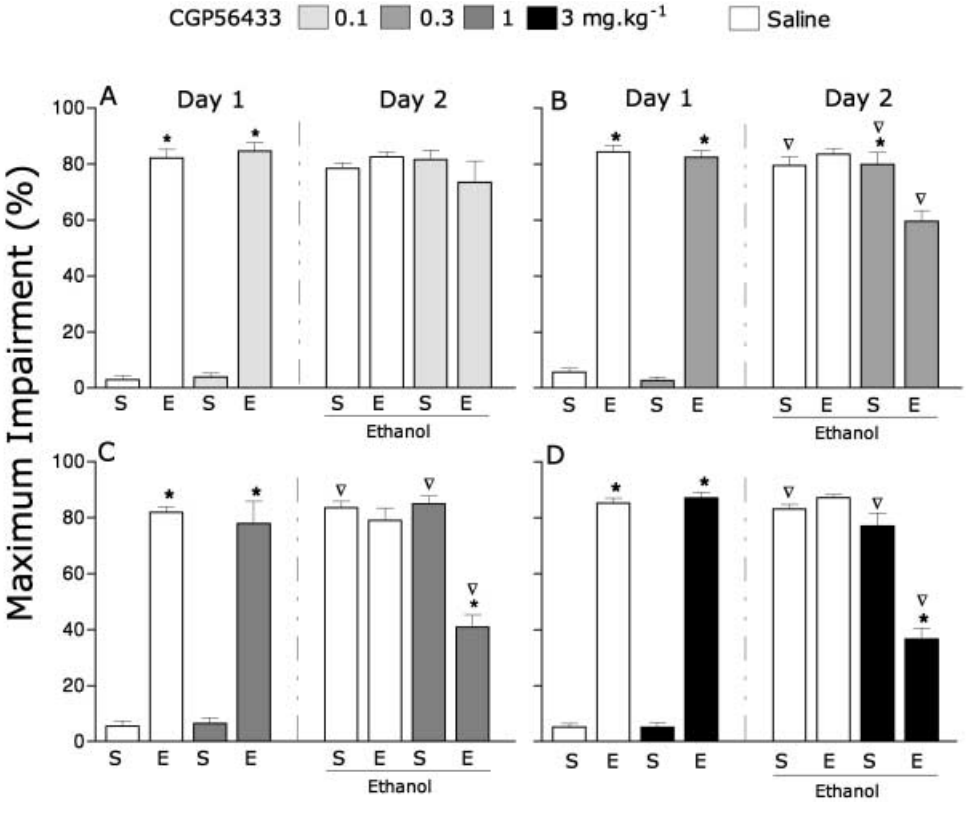


**Fig. 4A–D** Influence of CGP56433 on the development of rapid tolerance to ethanol-induced motor impairment. On day 1, control groups received saline (*S*) and experimental groups received CGP56433 (0.1, 0.3, 1.0, or 3.0 mg kg$^{-1}$; **A–D**, respectively) 30 min prior to saline or ethanol (*E*; 1.75 g kg$^{-1}$), and were then tested on the rota-rod. After 24 h, all animals were injected with ethanol (1.75 g kg$^{-1}$) and tested on the rota-rod to evaluate rapid tolerance (day 2). Results shown are means ± SEM of ten animals per group. * $P$<0.05 compared to respective control on the same day (Tukey's test), ▽ $P$<0.05 compared to performance of the same group on the previous day

[Fig. 4B: $F(1,36)=17.48$, $P$<0.001; Fig. 4C: $F(1,36)=13.27$, $P$<0.001; Fig. 4D: $F(1,36)=56.36$, $P$<0.0001], as well as a significant pretreatment×treatment×day interaction (Fig. 4B: $F(1,36)=8.34$, $P$<0.01; Fig. 4C: $F(1,36)=8.86$, $P$<0.01; Fig. 4D: $F(1,36)=45.42$, $P$<0.0001).

### Influence of the pretreatment with CGP36742 or CGP56433 on the blockade of rapid tolerance to ethanol by (-)-baclofen

Figures 5A, B and 6A–C illustrate the influence of CGP36742 (3 or 10 mg kg$^{-1}$) or CGP56433 (0.3, 1.0, or

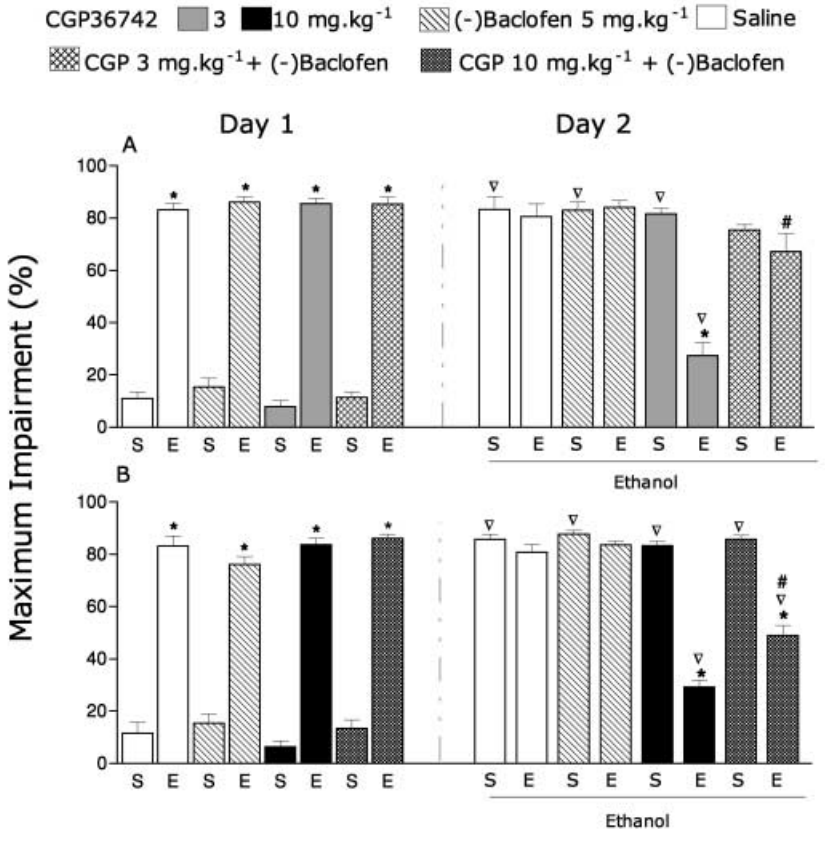


**Fig. 5A,B** Influence of CGP36742 followed by (-)-baclofen, on the development of rapid tolerance to ethanol-induced motor impairment. On day 1, control groups received saline (*S*) and experimental groups received CGP36742 (3 or 10 mg kg$^{-1}$; **A, B**, respectively). After 30 min control groups were pretreated with saline or (-)-baclofen (5 mg kg$^{-1}$). Twenty minutes later, the animals received saline or ethanol (*E*; 1.75 g kg$^{-1}$, i.p.), and were then tested on the rota-rod. After 24 h, all animals were injected with ethanol (1.75 g kg$^{-1}$, i.p.) and tested on the rota-rod to evaluate rapid tolerance (day 2). Results shown are means ± SEM of ten animals per group. * $P$<0.05 compared to respective control on the same day, ▽ $P$<0.05 compared to performance of the same group on the previous day, # $P$<0.05 compared to performance on the same day of group pretreated (on day 1) with CGP36742 10 mg kg$^{-1}$ alone and treated with ethanol (Tukey's test)

3.0 mg kg$^{-1}$) pretreatment, respectively, on the ability of (-)-baclofen (5 mg kg$^{-1}$,) to alter the development of rapid tolerance to ethanol. Considering the influence of CGP36742, the ANOVA revealed a significant effect of pretreatment with the $GABA_B$ receptor antagonist [Fig. 5A: $F(1,72)=40.15$, $P<0.0001$; Fig. 5B: $F(1,72)=31.92$, $P<0.0001$], of treatment with (-)-baclofen [Fig. 5A: $F(1,72)=13.03$, $P<0.001$], and of treatment with ethanol [Fig. 5A: $F(1,72)=287.68$, $P<0.0001$; Fig. 5B: $F(1,72)=203.20$, $P<0.0001$]. ANOVA revealed also a pretreatment×treatment×day interaction [Fig. 5A: $F(1,72)=10.95$, $P<0.01$]. The post hoc analysis showed that both doses of CGP36742 each facilitated the development of rapid tolerance to ethanol, confirming the data obtained in the previous experiments (Tukey's test). (-)-Baclofen 5 mg kg$^{-1}$, which alone did not affect rota-rod performance on day 2, completely blocked the development of rapid tolerance to ethanol when coadministered on day 1 with 3 mg kg$^{-1}$ CGP36742. However, this blockade by (-)-baclofen was only partial when the dose of CGP36742 was increased to 10 mg kg$^{-1}$, since comparisons between groups treated with CGP36742 (10 mg kg$^{-1}$) + ethanol and CGP36742 (10 mg kg$^{-1}$) followed by (-)-baclofen (5 mg kg$^{-1}$) + ethanol were also significant (Tukey's test).

The results obtained with the association between CGP56433 and (-)-baclofen on ethanol tolerance are depicted in Fig. 6. On day 2, groups pretreated only with CGP56433 and ethanol on the previous day developed rapid tolerance to ethanol. The previous treatment with (-)-baclofen, however, blocked the development of tolerance stimulated by 1 mg kg$^{-1}$ CGP56433. The facilitating effect of the higher doses of this $GABA_B$ receptor antagonist was not significantly affected by (-)-baclofen. ANOVA of the data revealed a significant effect of treatment with ethanol [Fig. 6A: $F(1,72)=294.33$, $P<0.0001$; Fig. 6B: $F(1,72)=257.72$, $P<0.0001$; Fig. 6C: $F(1,72)=325.10$, $P<0.0001$]. There was also a significant effect of pretreatment with (-)-baclofen [Fig. 6A: $F(1,72)=12.44$, $P<0.001$; Fig. 6C: $F(1,72)=8.70$, $P<0.01$] and with CGP56433 [Fig. 6B: $F(1,72)=63.11$, $P<0.0001$; Fig. 6C: $F(1,72)=24.90$, $P<0.0001$]. Post hoc analysis showed that CGP56433 facilitated the development of tolerance to ethanol and that (-)-baclofen could interfere with this effect. Taken together, these results suggest that the treatment with (-)-baclofen (5 mg kg$^{-1}$) blocked the facilitating effect of CGP36742 (3 mg kg$^{-1}$) and of CGP56433 (0.3 mg kg$^{-1}$) on the development of rapid tolerance to ethanol.

The determination of ethanol concentrations in blood taken from animals pretreated with saline, (-)-baclofen, CGP36742, or CGP56433 did not reveal any statistical differences between groups (data not shown).

## Discussion

It is quite well established that $GABA_A$ receptors are involved in ethanol actions (Cott et al. 1976; Karcz-Kubicha and Liljequist 1995; Liljequist and Engel 1982;

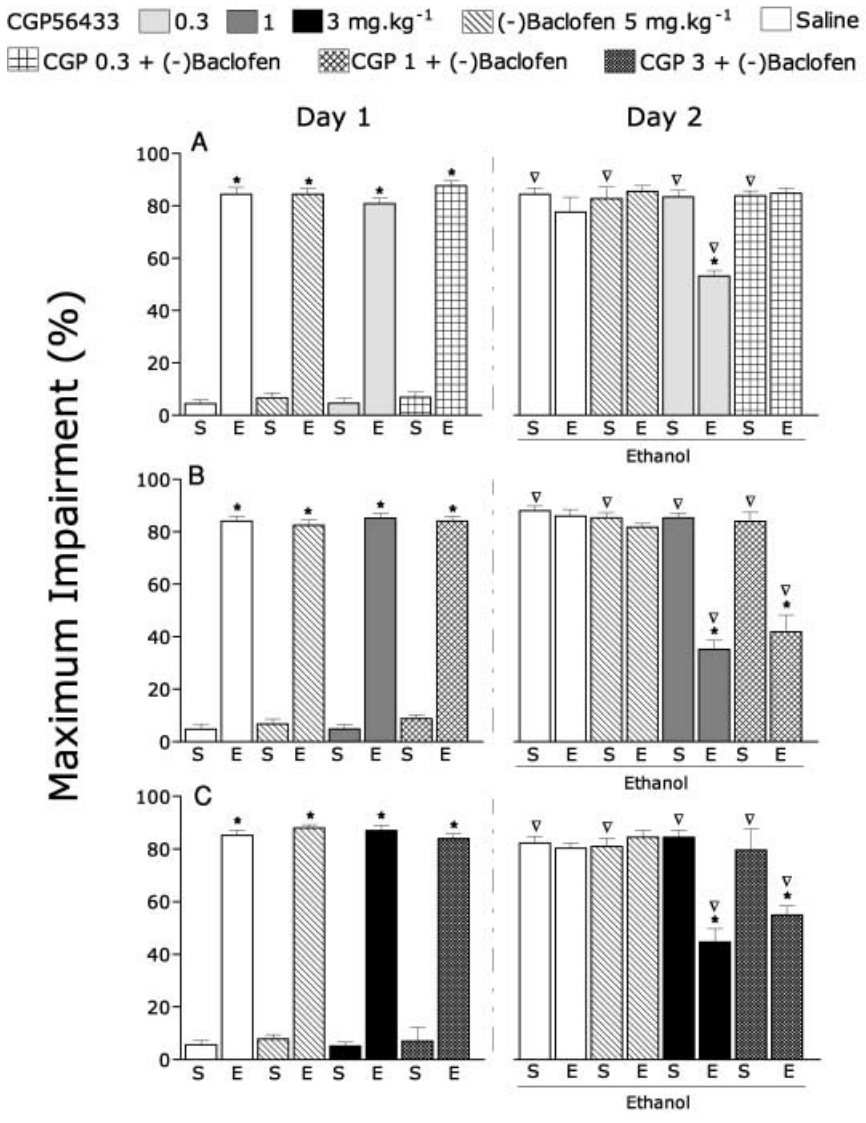


**Fig. 6A–C** Influence of CGP56433 followed by (-)-baclofen, on the development of rapid tolerance to ethanol-induced motor impairment. On day 1, control groups received saline (*S*) and experimental groups received CGP56433 (0.3, 1, or 3 mg $kg^{-1}$; **A–C**, respectively). After 30 min control groups were pretreated with saline or (-)-baclofen (5 mg $kg^{-1}$). Twenty minutes later, the animals received saline or ethanol (*E*; 1.75 g $kg^{-1}$), and were then tested on the rota-rod. After 24 h, all animals were injected with ethanol (1.75 g $kg^{-1}$) and tested on the rota-rod to evaluate rapid tolerance (day 2). Results shown are means ± SEM of ten animals per group. * $P<0.05$ compared to respective control on the same day (Tukey's test), ▽ $P<0.05$ compared to performance of the same group on the previous day

Mehta and Ticku 1990; Reynolds et al. 1992), but very little evidence has accumulated to date as to the possible contribution of $GABA_B$ receptor-mediated mechanisms to such effects. The results of the present study suggest that drugs acting upon $GABA_B$ receptors can affect rapid tolerance to ethanol in mice.

We used the rota-rod apparatus to evaluate the animals' performance, since this is a reliable method in the analysis of motor impairment (Bogo et al. 1981; Hoffmann and Tabakoff 1984; Jones and Roberts 1968; Szabó et al. 1994). Motor coordination tests are widely used to evaluate tolerance, either alone or compared to other tests. For example, similar results were obtained in studies on the influence of NMDA receptor-acting drugs on ethanol tolerance in tests involving motor coordination or physiological responses (Khanna et al. 1991, 1993, 1997). However, a task-dependent tolerance to ethanol after combined treatment with arginine-vasopressin plus ethanol has also been observed (Wu et al. 1996).

In the first experiment, a dose of ethanol sufficient to induce tolerance by itself was chosen, based on our previous work (Barreto et al. 1998). Administration of (-)-baclofen (5 and 7 mg $kg^{-1}$) on day 1 significantly blocked the development of rapid tolerance induced by ethanol evaluated on day 2. The higher dose of (-)-baclofen also induced muscular relaxation, as reflected by the decrease in motor performance it caused on day 1. Since no residual effect of pretreatment administered on day 1 was observed on the basal performance of animals evaluated on day 2, it seems that this incoordinating effect of (-)-baclofen does not interfere with the blockade of tolerance induced by the $GABA_B$ receptor agonist. In fact, a cross-tolerance between ethanol and (-)-baclofen would be expect in that case. It is interesting to mention that (-)-baclofen was shown to block the development of sensitization to ethanol (Broadbent and Harless 1999) and to other drugs (Kalivas and Stewart 1991).

Conversely, the $GABA_B$ receptor antagonists CGP36742 and CGP56433 facilitated the acquisition of

rapid tolerance using a total dose of ethanol on day 1 that did not induce tolerance per se (2.75 mg $kg^{-1}$). CGP56433 (0.3 and 1 mg $kg^{-1}$) facilitated the development of rapid tolerance at doses tenfold lower than the doses of CGP36742 (3 and 10 mg $kg^{-1}$). These data are in agreement with in vitro results showing that CGP56433 is more potent than CGP36742 in depressing GABA-induced late inhibitory postsynaptic potentials and in depressing the paired-pulse widening of excitatory postsynaptic potentials in the CA1 region of hippocampal slices (Pozza et al. 1999). CGP36742 (30 mg $kg^{-1}$) antagonized the motor impairment induced by ethanol on day 1 but did not affect tolerance. This could be related to the fact that a certain level of motor impairment on day 1 is required for the development of rapid tolerance on day 2 in this model (Barreto et al. 1998).

The association of (-)-baclofen plus the $GABA_B$ receptor antagonists, both given on day 1, revealed that (-)-baclofen abolished the effect of the lower doses of both $GABA_B$ receptor antagonists and partially blocked the effects of the higher dose of CGP36742, but did not block the stimulating effect of CGP56433 (1 and 3 mg $kg^{-1}$) on tolerance. The effects of the $GABA_B$ receptor agonist and antagonists did not seem to be due to pharmacokinetic interactions with ethanol, since blood ethanol levels of animals pretreated with saline, (-)-baclofen, CGP36742, or CGP56433 (or associations of these drugs) did not differ significantly. Although peripheral blood was taken, brain and plasma ethanol levels measured at the same time after ethanol i.p. administration are similar (Gostomzyk et al. 1969; Tabakoff et al. 1986). Also, even though the determination of blood ethanol levels at different times after injection could be more elucidative in respect to the influence of $GABA_B$ receptor-acting drugs on ethanol pharmacokinetics, we decided to evaluate blood ethanol levels at the time of maximum motor impairment in all groups. Moreover, a recent study with the same range of doses of (-)-baclofen we used showed that this drug did not influence blood ethanol levels at 15, 50, and 100 min after ethanol injections (Broadbent and Harless 1999).

It is known that acute administration of the $GABA_B$ receptor antagonist phaclofen reduces motor impairment, hypothermia, and the motor activity induced by ethanol (Allan and Harris 1989) as well as the anticonvulsant effect of ethanol (Mehta and Ticku 1990). Moreover, $GABA_B$ receptor agonists or antagonists influenced, in opposite ways, withdrawal signs of mice submitted to discontinuation of chronic ethanol treatment (Mead and Little 1995). Although the possibility of a non-specific additive effect of the $GABA_B$ receptor agonist (or a non-specific subtractive effect of the $GABA_B$ receptor antagonist) with ethanol can not be ruled out, we did not find any statistical differences between ethanol groups pretreated with saline or drugs acting at $GABA_B$ receptors. Therefore, the influence of $GABA_B$ receptor-acting drugs on rapid tolerance does not seem to be a consequence of a direct influence on ethanol's actions in the brain. Since (-)-baclofen, CGP36742, and CGP56433 were injected only on the 1st day of the experiment and that the absolute basal performance of mice evaluated on day 2 was unaffected, the result on day 2 seems to be a consequence of some action of agonist and antagonist drugs that occurred on the previous day.

The involvement of another neurotransmitter system could also explain the influence of the $GABA_B$ receptor-acting drugs. Tolerance may be influenced by several neurotransmitter systems, such as GABA, serotonin, arginine-vasopressin, and acetylcholine (Kalant 1996; Wu et al. 1994, 1996). Furthermore, recently Chandler et al. (1998) have proposed that multiple mechanisms are involved in the development of ethanol tolerance, including both transcriptional and post-translational modifications in NMDA and $GABA_A$ receptors.

In the last three decades, several studies have suggested that learning factors are important to the development of tolerance, thus changing its classic concept (Bitrán and Kalant 1991; Chen 1968; Holloway et al. 1989; Kalant 1998; Leblanc et al. 1973, 1975). GABAergic activity appears to play a fundamental role in long-term potentiation induction, as increased GABA release is associated with enhanced inhibition of the NMDA complex, whereas a reduction in GABA release exacerbates glutamatergic activity, and facilitates learning and memory retention (Collingridge and Singer 1990; Collingridge et al. 1992; Peris et al. 1997; Recasens 1995). It was shown that the $GABA_B$ receptor agonist (-)-baclofen reduced, and that some $GABA_B$ receptor antagonists including CGP36742 increased, endogenous glutamate release (Teoh et al. 1996). $GABA_B$ receptors have also been shown to modulate the glutamate-mediated secretion of the regulatory neuropeptides vasopressin and oxytocin (Kombian et al. 1996). Considering that (-)-baclofen dose-dependently impairs spatial learning in rats (McNamara and Skelton 1996) whereas CGP36742 increases memory retention in different animal models (Carletti et al. 1993; Mondadori et al. 1996), the current results could be interpreted to suggest that the influence of $GABA_B$ receptor-acting drugs on the development of rapid tolerance to ethanol could be related to interference with learning and memory mechanisms, probably through an interaction with the NMDA system. However, further studies will be needed to consolidate this view. Nevertheless, taken together, the present results provide clear behavioral evidence for a role for $GABA_B$ receptors in the development of rapid tolerance to ethanol.

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