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γ-Hydroxybutyric Acid and Diazepam Antagonize a Rapid Increase in $GABA_A$ Receptors α_4 Subunit mRNA Abundance Induced by Ethanol Withdrawal in Cerebellar Granule Cells

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ABSTRACT

Both benzodiazepines and γ-hydroxybutyric acid (GHB) are used to treat alcohol withdrawal syndrome. The molecular basis for this therapeutic efficacy was investigated with primary cultures of rat cerebellar granule cells. Long-term exposure of these cells to ethanol (100 mM, 5 days) reduced the abundance of mRNAs encoding the γ_2L and γ_2S subunits of the GABA type A receptor (−32 and −23%, respectively) but failed to affect that of α_1, α_4, or α_6 subunit mRNAs. Subsequent ethanol withdrawal resulted in decreases in the amounts of α_1 (−29%), α_6 (−27%), γ_2L (−64%), and γ_2S (−76%),subunit mRNAs that were maximal after 6 to 12 h. In contrast, 3 h after ethanol withdrawal, the abundance of the α_4 subunit mRNA was increased by 46%. Ethanol withdrawal did not affect neuronal morphology but reduced cellular metabolic activity. The increase in α_4 subunit was confirmed by functional studies showing a positive action of flumazenil in patch clamp recordings of GABA-stimulated currents after ethanol withdrawal. Diazepam (10 μM) or GHB (100 mM) prevented the increase in the amount of the α_4 subunit mRNA, the metabolic impairment, and the positive action of flumazenil induced by ethanol withdrawal but failed to restore the expression of the α_1 and γ_2 subunits. The antagonism by GHB seems not to be mediated by a direct action at $GABA_AR$ because GHB failed to potentiate the effects of GABA or diazepam on Cl^- currents mediated by GABA type A receptor.

Ethanol elicits its central effects through modulation of neurotransmission mediated by various receptors, especially that mediated by GABA type A receptors ($GABA_AR$) (Crews et al., 1996; Mehta and Ticku, 1999a). $GABA_AR$ are heterogeneous in that they comprise various combinations of subunits (Barnard et al., 1998). The absence or presence of particular α subunit isoforms in these receptors confers selectivity for certain drugs (Barnard et al., 1998). Different α subunits also mediate distinct pharmacological actions of benzodiazepines, including sedative-hypnotic (Rudolph et al., 1999), anxiolytic, and myorelaxant (Low et al., 2000) effects. Long-term treatment either of rats or of cultured neurons with drugs that modulate GABAergic function, such as benzodiazepines (Holt et al., 1996; Follesa et al., 2001), barbiturates (Tyndale et al., 1997), and steroids (Yu et al., 1996; Concas et al., 1998; Follesa et al., 1998; Smith et al., 1998a,b; Follesa et al., 2000) affects the expression of the genes for various $GABA_AR$ subunits. Long-term ethanol administration also affects the subunit composition and, consequently the functional properties, of native $GABA_AR$ (Morrow et al., 1990; Mhatre et al., 1993; Devaud et al., 1997). The pharmacological profile of ethanol is highly similar to that of benzodiazepines. Long-term exposure to ethanol, like that to benzodiazepines, also results in the development of tolerance and dependence. The precise molecular mechanism by which prolonged ethanol consumption modifies $GABA_AR$ function, however, has remained unknown.

Benzodiazepines are among the safest and most effective drugs used in the treatment of alcohol withdrawal syndrome. γ-Hydroxybutyric acid (GHB), a GABA metabolite naturally present in the brain that in pharmacological doses modulates a variety of neurotransmission systems (Gessa et al., 1968; Bernasconi et al., 1999), has also been proposed for use in the treatment of persons with this condition (Gallimberti et al., 1992; Addolorato et al., 1998). Although specific recognition sites for GHB have been identified in the brain (Benavides et al., 1982), it remains unclear whether all the effects of this compound are mediated by these sites. GHB reduces the self-administration of alcohol and suppresses alcohol with-

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ABBREVIATIONS: $GABA_AR$, GABA type A receptors; GHB, γ-hydroxybutyric acid; PCR, polymerase chain reaction; MBS, modified Barth's solution; ANOVA, analysis of variance.

drawal signs in alcohol-preferring rats (Fadda et al., 1989). In humans, a single dose of GHB has been shown to suppress alcohol withdrawal symptoms for several hours, and repeated administration increases the number of days of abstinence from alcohol and reduces the number of drinks per day (Gallimberti et al., 1992). However, the molecular mechanism responsible for these effects is not known.

We have recently shown that long-term exposure to and subsequent withdrawal of benzodiazepines, zaleplon, zolpidem, or neurosteroids result in selective changes in the expression of specific $GABA_AR$ mRNA and polypeptide subunits and in $GABA_AR$ function in cultured cerebellar granule cells (Follesa et al., 2000, 2001, 2002). In particular, discontinuation of long-term treatment of the cultured neurons with diazepam both resulted in a selective increase in the abundance of the α_4 subunit mRNA and polypeptide and prolonged a decrease in the amounts of the α_1 and γ_2 subunit mRNA and corresponding protein that was already apparent during long-term drug exposure (Follesa et al., 2001). These changes in mRNA and corresponding protein produced changes in receptor function (Follesa et al., 2001). Long-term diazepam administration produced a reduction in the efficacy of this drug in potentiating the GABA-evoked Cl^- currents (Follesa et al., 2000, 2001). In the same article, we demonstrated that withdrawal from diazepam or imidazenil was associated with both a reduced ability of diazepam to potentiate GABA action and the ability of flumazenil to potentiate GABA action. This effect of flumazenil in withdrawal cells resulted from the increase of the α_4 subunit mRNA and corresponding protein (Follesa et al., 2000, 2001).

Given that long-term ethanol administration and withdrawal elicit neurochemical and molecular effects similar to those induced by drugs able to activate $GABA_AR$ (Morrow et al., 1990; Mhatre et al., 1993; Devaud et al., 1997), we have now studied primary cerebellar granule cell cultures subjected to abrupt discontinuation of ethanol treatment and evaluated the effects of diazepam and GHB during ethanol withdrawal on the gene expression and function of the $GABA_AR$. The use of this exemplified model system will provide new insights that might help to understand, the role played by single subunits of the $GABA_AR$ during withdrawal.

Materials and Methods

Cell Culture. Primary cultures of cerebellar neurons enriched in granule cells were prepared from cerebella of 8-day-old rats. After culture for 8 days, these cells contain the mRNAs for all 14 subunits of the $GABA_AR$ with an expression pattern similar to that apparent in the postnatal developing cerebellum but different from that observed in the adult rat cerebellum. Cells were plated (12.5×10^6 cells in 10 ml per dish) in 100-mm dishes that had been coated with poly(L-lysine) (10 μg/ml; Sigma, St. Louis, MO). For the electrophysiological recording, cells were plated (3×10^5 cells in 1 ml) in multiwell plates containing, in each well, 12-mm round coverslips coated with poly(L-lysine). Cells from either type of plating were cultured in basal Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM glutamine, gentamicin (100 μg/ml; Sigma), and 25 mM KCl. Such a high concentration of potassium was necessary to induce a persistent depolarization, which promotes the survival of granule cells. Cytosine arabinofuranoside (final concentration, 10 μM; Sigma) was added to the cultures 18 to 24 h after plating to inhibit the proliferation of non-neuronal cells.

After 3 days in culture, the cells were exposed for 5 days to ethanol at the indicated concentrations. In some ethanol-withdrawal experiments, the medium containing ethanol was then replaced with ethanol-free medium containing GHB (at the indicated concentrations) or diazepam (10 μM). Ethanol was diluted in medium, GHB was dissolved in medium, and diazepam was dissolved in dimethyl sulfoxide and subsequently diluted in medium. Control cells were treated with the corresponding vehicle. The culture medium was replaced every day with fresh medium containing the indicated drug.

Probe Preparation. The cDNA for each subunit of the $GABA_AR$ studied was prepared as described previously (Follesa et al., 1998) by reverse transcription and polymerase chain reaction (PCR). In brief, cDNA prepared from rat brain (1–10 ng) was amplified by PCR with *Taq*DNA polymerase (2.5 U; PerkinElmer, Boston, MA) in 100 μl of standard buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin) containing 1 μM each of specific sense and antisense primers and 200 μM of each deoxynucleoside triphosphate. The primer pairs for the various subunits of the $GABA_AR$ were designed to include cDNA sequences with the lowest degree of intersubunit homology (Follesa et al., 1998). The primers used to amplify the α_6 subunit cDNA were 5′-GGGAAAAAGTCAATTGCTCAC-3′ and 5′-CTCCTTATTAATCC-3′ (upstream and downstream, respectively). The reaction was performed in a thermal cycler (Eppendorf) for 30 cycles of 94°C for 45 s, 60°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 15 min. The PCR products were separated by electrophoresis, visualized by staining with ethidium bromide, excised from the gel, purified, and cloned into the pAMP 1 cloning vector (Invitrogen). *Escherichia coli* DH5α was transformed with the resulting plasmids, which were subsequently purified from the bacteria and the cDNA inserts were sequenced with a Sequenase DNA sequencing kit (USB, Cleveland, OH). The determined nucleotide sequences were 100% identical to the respective previously published sequences. Plasmids containing the cDNA fragments corresponding to the various $GABA_AR$ subunits were linearized with restriction enzymes (Follesa et al., 1998) and then used as templates, together with the appropriate RNA polymerase (SP6 or T7) to generate [α-^{32}P]UTP-labeled cRNA probes for RNase protection assays.

RNA Extraction and Measurement of $GABA_AR$ Subunit mRNAs. Total RNA was isolated from cultured cerebellar granule cells by the guanidine isothiocyanate method as described previously (Follesa et al., 1998) and quantified by measurement of absorbance at 260 nm. An RNase protection assay for the semiquantitative detection of the $GABA_AR$ α_1, α_4, α_6, γ_2L, and γ_2S subunit mRNAs was performed as described previously (Follesa et al., 1998). In brief, 25 μg of total RNA was dissolved in 20 μl of hybridization solution containing 150,000 cpm of ^{32}P-labeled cRNA probe for a specific $GABA_AR$ subunit mRNA (specific activity, 6×10^7 to 7×10^7 cpm/μg) and 15,000 cpm of ^{32}P-labeled cyclophilin cRNA (1×10^6 cpm/μg). Given that cyclophilin is expressed widely among tissues, including the brain, and that its gene is most likely regulated in an "on or off" manner, we used cyclophilin mRNA as an internal standard for our measurements (Follesa et al., 1998). The hybridization reaction mixture was incubated overnight at 50°C and then subjected to digestion with RNase, after which RNA-RNA hybrids were detected by electrophoresis (on a sequencing gel containing 5% polyacrylamide and urea) and autoradiography. The amounts of $GABA_AR$ subunit mRNA and cyclophilin mRNA were determined by measuring the optical density of the corresponding bands on the autoradiogram with a densitometer (GS-700; Bio-Rad, Hercules, CA); this instrument is calibrated to detect saturated values, so that all our measurements were in the linear range. The data were normalized by dividing the optical density of the protected fragment for each receptor subunit mRNA by that of the respective protected fragment for cyclophilin mRNA. The amount of each receptor subunit mRNA was therefore expressed in arbitrary units.

Metabolic Activity of Cerebellar Granule Cells. The metabolic activity of live cerebellar granule cells was measured with the resazurin (TOX-8) system (Magnani and Bettini, 2000). Resazurin (Sigma) is a dye that is blue in its oxidized form and red in

its reduced form. Bioreduction of the dye by viable cells can thus be monitored spectrophotometrically and provides an indicator of cellular energy status. Cerebellar granule cells (7×10^5) were cultured in 24-well plates coated with poly(L-lysine) and containing 1 ml of minimum essential medium devoid of phenol red (Invitrogen) per well. They were treated for 5 days with ethanol (100 mM) and then subjected to ethanol withdrawal in the absence or presence of GHB or diazepam as described above. Four replica wells were used for each treatment. After ethanol withdrawal for 3 or 6 h, 100 μl of TOX-8 stock solution was added to each well, and the plate was incubated for 2 h in the dark under standard conditions (37°C, humidified atmosphere containing 5% CO_2). The absorbance of the dye was then measured at wavelengths of 600 and 690 nm. The absorbance of blank wells containing culture medium and the appropriate drug but lacking cells was also determined. The mean value of blank wells was subtracted from that of the experimental wells to yield net absorbance values. Metabolic activity was determined as the change in absorbance caused by resazurin reduction, and data are expressed as percentage change in metabolic activity relative to that of control cells (not treated with ethanol). To verify that changes in metabolic activity were not caused by cell death, we also counted with a hemocytometer the number of viable cells in each well after their removal with trypsin and staining with trypan blue.

Whole-Cell Patch-Clamp Electrophysiological Recording. Immediately before recording, coverslips were transferred to a perfusion chamber (Warner Instruments, Hampden, CT), and cerebellar granule cells were visualized under a Nikon upright microscope equipped with Nomarski optics. Membrane potentials were clamped at −60 mV with a Axopatch 200-B amplifier (Axon Instruments, Union City, CA). The resting membrane potential for the recorded neurons was approximately −60 mV. Recording pipettes (borosilicate capillaries with filament, outer diameter 1.5 mm; Sutter Instruments, Novato, CA) were prepared with a two-step vertical puller (Sutter Instruments) and had resistances between 4 and 6 MΩ. Pipette capacitance and series resistances were compensated, the latter at 60%. Currents through the patch-clamp amplifier were filtered (eight-pole bessel, 2 kHz) and digitized at 5.5 kHz using commercial software (pClamp 8.1; Axon Instruments).

The external solution contained 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.3, and 11 mM glucose (all chemicals from Sigma). The internal solution contained 140 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES, pH 7.3, and 2 mM 5′-ATP-Na_2 (all chemicals from Fluka, Buchs, Switzerland). Drugs were applied with a fast-exchange flow-tube perfusion system driven by motor (Warner Instruments Co.). GABA was applied at a concentration of 1 to 3 μM, which induced a current with an amplitude of 5 to 10% of the maximal response (EC_{5-10}). Flumazenil (3 μM) was applied at 30-s intervals. All experiments were performed at room temperature (23–25°C). Data were analyzed by pClampFit 8.01 (Axon Instruments, Union City, CA). Modulation of GABA-evoked Cl^- currents by flumazenil is presented as percentage change, $[(I'/I) - 1] \times 100\%$, where I is the average of control responses obtained before application and after washout of drugs, and I' is the average of agonist-induced response obtained from the same cell in the presence of drugs.

Electrophysiological Recording Cloned $GABA_AR$. The human α_1, β_2, and γ_2L subunit cDNAs were subcloned into the pCDM8 vector (Invitrogen) for nuclear injection. Oocytes were isolated from *Xenopus laevis* as described. A mixture of the three subunit cDNAs (0.5 ng each in a total volume of 30 nl) was injected into the animal pole of each oocyte (Colman, 1984). One to 4 days after injection, oocytes expressing recombinant $\alpha_{1\beta2\gamma2}$L receptors were placed in a chamber (volume, ~100 μl) and perifused (2 ml/min) with modified Barth's solution (MBS) comprising 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES-NaOH, pH 7.5, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, and 0.91 mM $CaCl_2$. GABA was dissolved in MBS and applied for 30 s. Diazepam was dissolved in dimethyl sulfoxide and then diluted in MBS; the final concentration of solvent in MBS was 1% and did not affect GABA responses. GHB was dissolved in H_2O and then diluted in MBS. Diazepam and GHB were each applied for 60 s before their coapplication for 30 s with a concentration of GABA that induced a current with an amplitude of 5 to 10% of the maximal response (EC_{5-10}).

Statistical Analysis. Data are presented as means ± S.E.M. and were subjected to analysis of variance (ANOVA) followed by Scheffé's F test. A p value of <0.05 was considered statistically significant.

Results

Effects of Long-Term Ethanol Treatment on $GABA_AR$ Subunit mRNA Abundance. Cerebellar granule cells were incubated for 5 days in the absence or presence of 10, 50, or 100 mM ethanol, after which the abundance of $GABA_AR$ α_1, α_4, α_6, γ_2L, and γ_2S subunit mRNAs was determined. Ethanol had no significant effect on the amounts of the α_1 (Fig. 1A), α_4 (Figs. 1B and 3), and α_6 (see below) subunit mRNAs. In contrast, ethanol induced a dose-dependent decrease in the abundance of the γ_2L and γ_2S splice variant mRNAs, with the amounts of these transcripts being reduced by 32 and 23%, respectively, after incubation of cells with 100 mM ethanol (Fig. 1, C and D).

Effects of Ethanol Withdrawal on $GABA_AR$ Subunit mRNA Abundance. We next investigated the effects of ethanol withdrawal on the abundance of $GABA_AR$ subunit mRNAs by incubating cerebellar granule cells first with 100 mM ethanol for 5 days and then in the absence of ethanol for 3, 6, 12, or 24 h. Ethanol withdrawal induced a decrease in the abundance of the α_1 subunit mRNA that was already significant after 3 h, maximal after 6 h (−29%), and no longer apparent at 24 h (Fig. 2A). Six hours after ethanol withdrawal, the abundance of the α_6 subunit mRNA was also significantly reduced [control cells, 100.0 ± 8.1%; cells treated with 100 mM ethanol for 5 days, 105.6 ± 2.6%; cells subjected to ethanol withdrawal for 6 h, 72.8 ± 2.6% ($p < 0.05$ versus control); data are means ± S.E.M. of values from three independent experiments]. Three hours after ethanol withdrawal, the abundance of the γ_2L and γ_2S subunit mRNAs remained decreased by extents similar to those apparent during long-term treatment. The amounts of these mRNAs declined further with time, achieving minimal values (36 and 24%, respectively) 12 h after ethanol withdrawal, before returning to control values at 24 h (Fig. 2, C and D). In contrast to the effects of ethanol withdrawal on the abundance of the α_1, α_6, and γ_2 subunit mRNAs, discontinuation of ethanol treatment induced a marked increase in the amount of the α_4 subunit mRNA (Figs. 2B and 3). This increase was maximal (+46%) 3 h after ethanol withdrawal, remained significant at 6 h, and was no longer apparent at 12 h.

Effect of Ethanol on Neuronal Metabolism. Neither treatment with 100 mM ethanol for 5 days nor subsequent ethanol withdrawal for 6 h seemed to affect the morphology of cerebellar granule cells (Fig. 4). In contrast, spectrophotometric measurement of resazurin reduction revealed that, whereas long-term ethanol treatment did not effect the metabolic activity of the cultured cells, ethanol withdrawal induced a time-dependent decrease in metabolic activity (Fig. 5A). This impairment in cellular metabolism was not attrib-

utable to cell death, given that the percentage of viable cells was not affected by either long-term ethanol treatment or ethanol withdrawal (Fig. 5B).

Effects of Diazepam and GHB on Ethanol Withdrawal. Exposure of cerebellar granule cells to diazepam (10 μM) at the time of ethanol withdrawal completely antagonized the withdrawal-induced increase in the abundance of the α_4 subunit mRNA (Fig. 6A). The replacement of ethanol with diazepam also blocked the ethanol withdrawal-induced impairment in cellular metabolism (Fig. 6B).

We also examined the effects of exposing cells to various concentrations (10 μM to 100 mM) of GHB at the time of ethanol withdrawal. GHB inhibited in a dose-dependent manner the increase in the abundance of the α_4 subunit mRNA induced by discontinuation of ethanol treatment. Only the highest concentration tested (100 mM) resulted in completely effective inhibition (Fig. 7A), whereas the concentration of 50 mM inhibited only partially but not significantly with a P value of 0.094234. At a concentration of 100 mM, GHB also completely prevented the ethanol withdrawal-induced impairment in cellular metabolism, whereas at a concentration of 50 mM, the inhibition was only partial but significant (Fig. 7B). In contrast, neither diazepam nor GHB, at concentrations that blocked the ethanol withdrawal-induced increase in the abundance of the α_4 subunit mRNA, had a significant effect on the

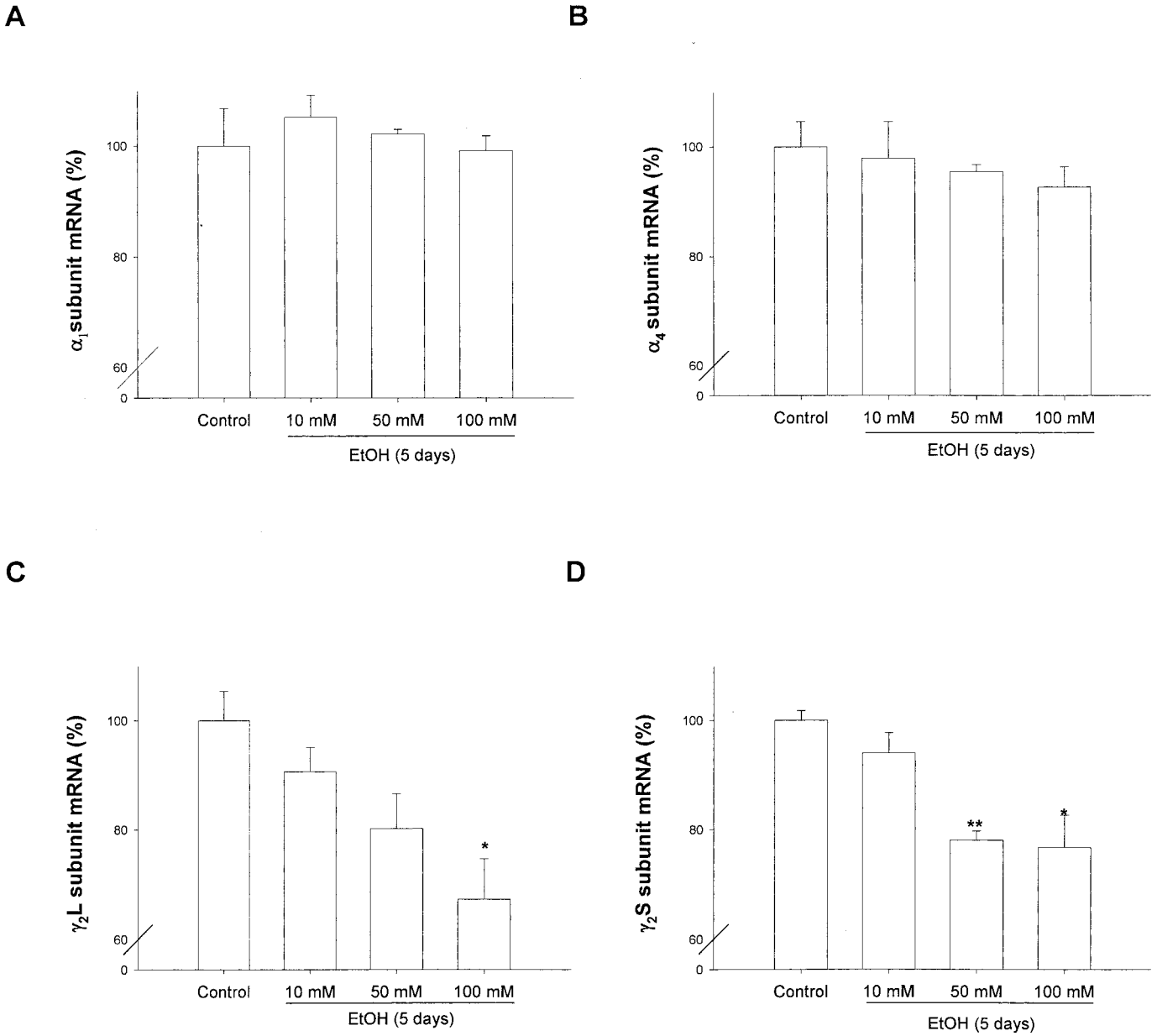

Fig. 1. Effects of long-term exposure to various ethanol concentrations on the abundance of $GABA_AR$ α and γ subunit mRNAs in cultured cerebellar granule cells. Cells were incubated for 5 days in the absence (control) or presence of 10, 50, or 100 mM ethanol, after which the amounts of $GABA_AR$ α_1 (A), α_4 (B), γ_2L (C), and γ_2S (D) subunit mRNAs were measured by RNase protection assay. Data are means ± S.E.M. of values from three independent experiments and are expressed as a percentage relative to control values. *, $p < 0.05$; **, $p < 0.01$ versus control (ANOVA and Scheffé's F test).

decrease in the abundance of the α_1 or γ_2 subunit mRNAs induced by ethanol withdrawal (Fig. 8).

Functional Characterization of $GABA_AR$ after Long-Term Ethanol Treatment and Withdrawal. To evaluate the functional consequences of the increase in α_4 subunit mRNA induced by ethanol withdrawal, we examined the ability of flumazenil in modulating the $GABA_AR$ function by patch-clamp electrophysiological recording of single cerebellar granule cells in culture. The modulatory action of flumazenil in granule cells that underwent extended treatment with ethanol was similar to that measured in control granule cells (Fig. 9). In contrast, in ethanol-withdrawn granule cells, 3 μM flumazenil potentiates (+53 ± 5%) the GABA-evoked Cl^- current [Fig. 9a result consistent with the ethanol withdrawal-induced up-regulation of the α_4 subunit in these cells (see Fig. 2 and 3)]. Finally, the substitution of 10 μM diazepam or 100 mM GHB for ethanol abolished the positive modulation of 3 μM

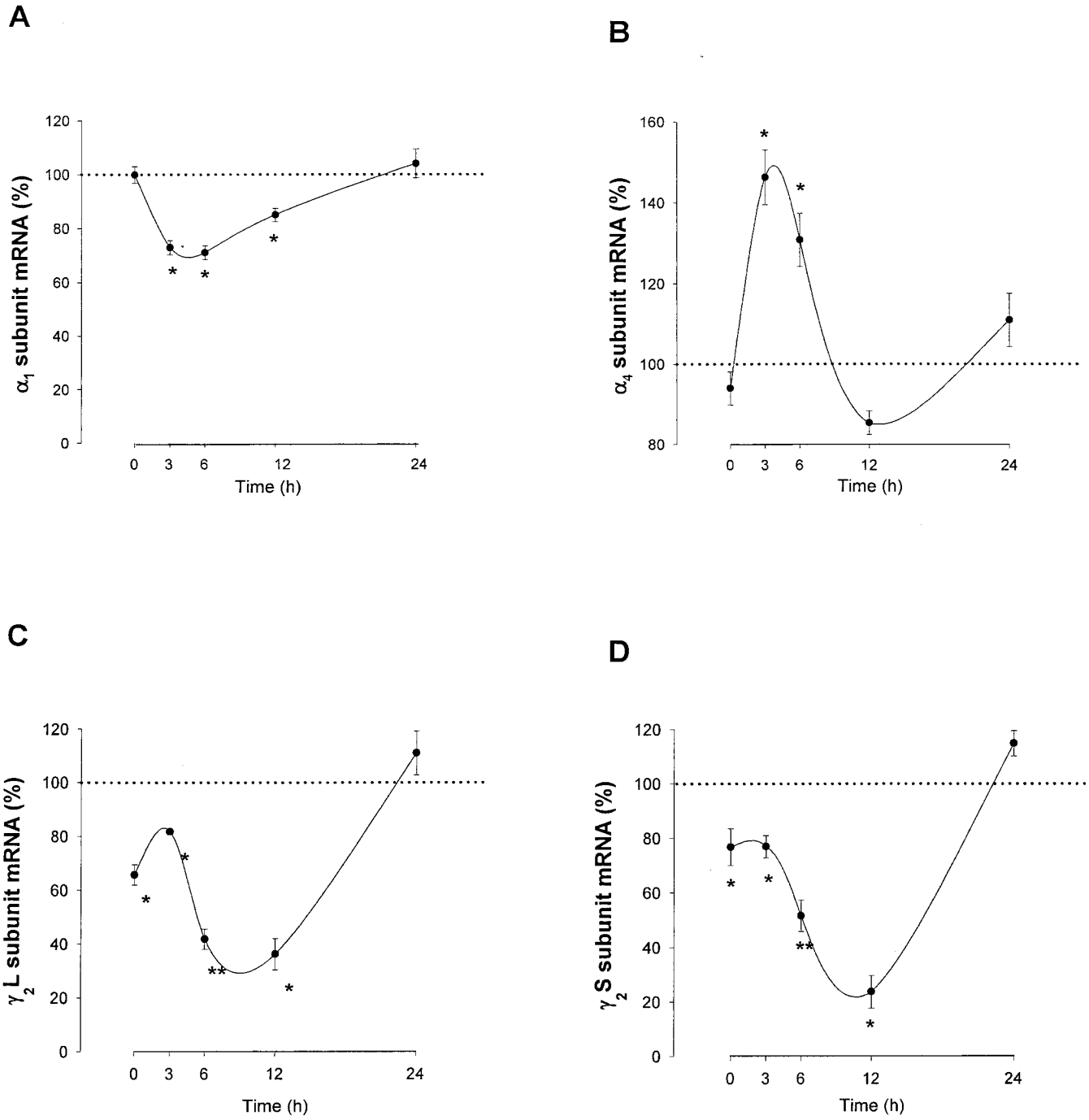

Fig. 2. Time course of the effects of ethanol withdrawal on the abundance of $GABA_AR$ subunit mRNAs in cerebellar granule cells. Cells were incubated first for 5 days with 100 mM ethanol and then for the indicated times in the absence of this agent. The amounts of $GABA_AR$ α_1 (A), α_4 (B), γ_2L (C), and γ_2S (D) subunit mRNAs were determined by RNase protection assay. Data are means ± S.E.M. of values from three independent experiments and are expressed as a percentage relative to the corresponding value for control cultures incubated in the absence of ethanol. *, $p < 0.05$; **, $p < 0.01$ versus control (ANOVA and Scheffé's F test).

flumazenil induced by ethanol withdrawal (Fig. 9). This finding is in agreement with the capability of these drugs to abolish the ethanol withdrawal-induced up-regulation of the α_4 subunit (see Figs. 6A and 7A).

Lack of Effect of GHB on $GABA_AR$ Function. Finally, we examined whether GHB affects the function of recombinant $\alpha_1\beta_2\gamma_2L$ $GABA_AR$ expressed in *X. laevis* oocytes. GHB (10 μM to 100 mM) had no effect on Cl^- currents induced by GABA at an $EC_{5\text{ to }10}$ (6 to 10 μM) (Fig. 10A). In the absence of GABA, GHB was also unable to activate directly the $GABA_AR$ complex at concentrations up to 100 mM (data not shown). Moreover, GHB (1 to 50 mM) failed to affect the enhancement of GABA-evoked Cl^- currents induced by 1 μM diazepam (Fig. 10B). These results were supported by our recent observation that

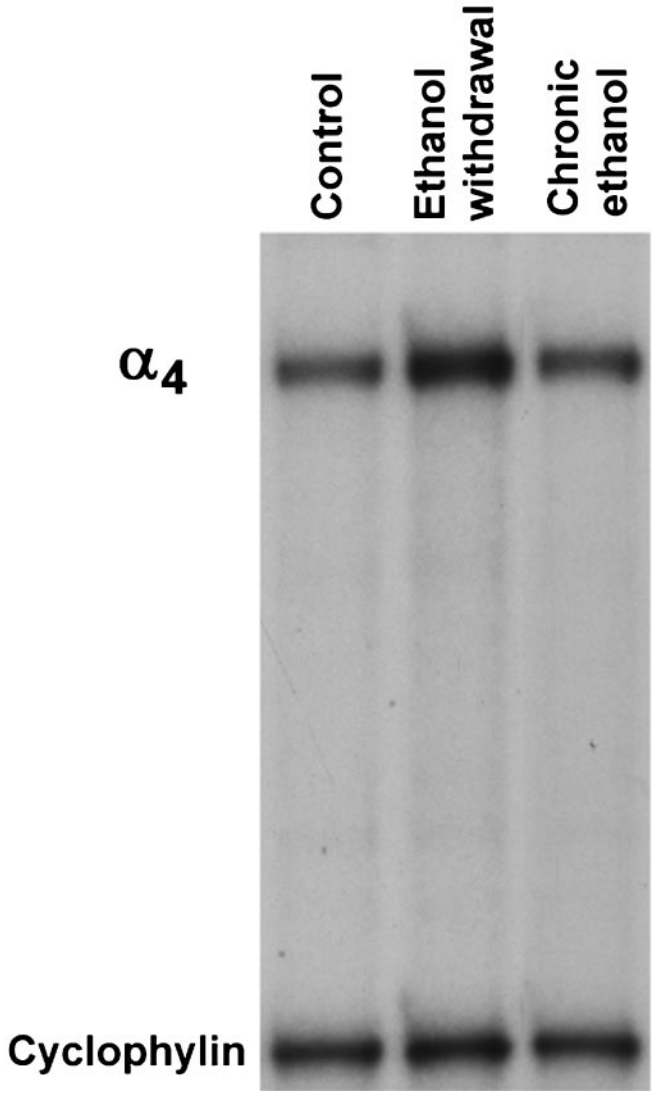

Fig. 3. $GABA_A$ receptor α_4 subunit mRNA detection by RNase protection assay, representative experiment. Autoradiograph showing positions of the $GABA_A$ receptor α_4 subunit and cyclophilin (internal standard) mRNAs on a urea/polyacrylamide electrophoresis gel. Cells were treated with ethanol (100 mM; 5 days) (chronic ethanol) or subject to 3h withdrawal (ethanol withdrawal). Control cells were treated with solvent. The total RNA was extracted (as described under *Materials and Methods*) from cerebellar granule cells 8 days in culture. On each lane, 25 μg of total RNA from individual plates were loaded. Ethanol withdrawal produced up-regulation of the α_4 subunit mRNA (+53 as calculated by densitometry in this autoradiogram), whereas chronic ethanol did not significantly modify the levels of the α_4 subunit mRNA (+7% as calculated by densitometry in this autoradiogram). The mean values of several experiments are shown in Fig. 2B.

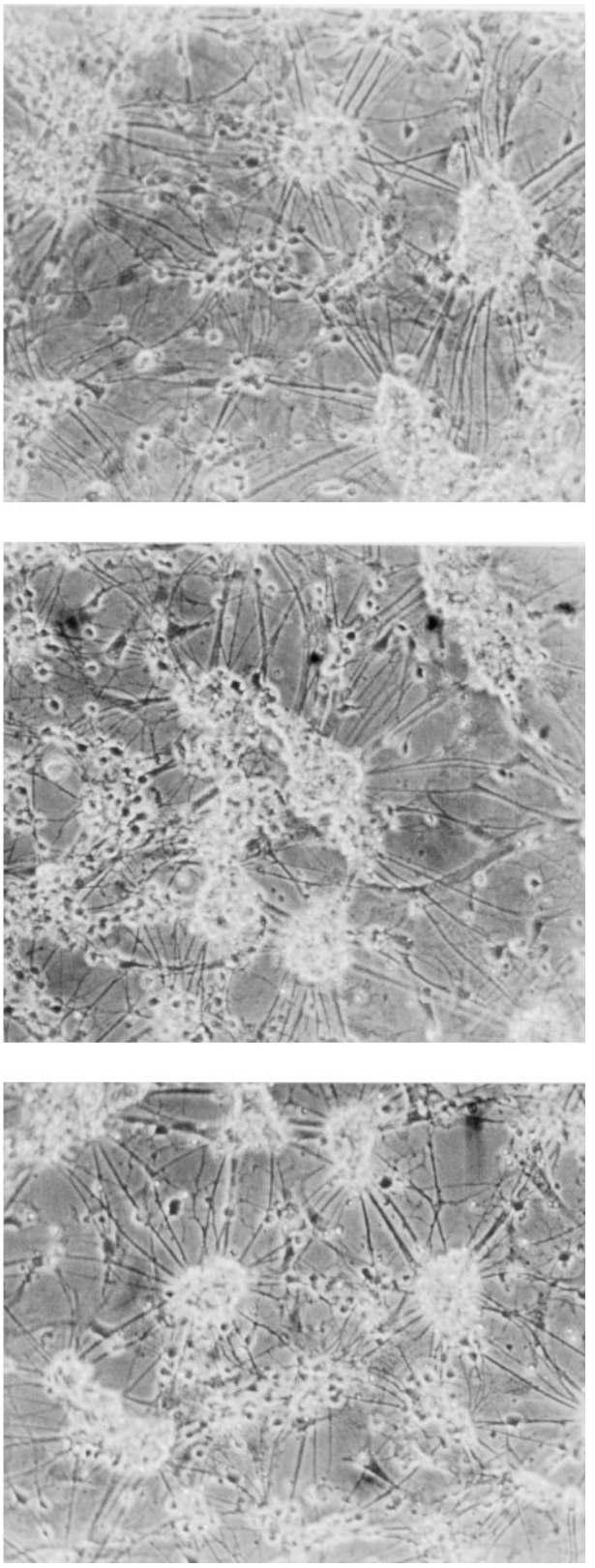

Fig. 4. Light micrographs of live rat cerebellar granule cells in culture. Top, control cells; middle, cells treated with 100 mM ethanol for 5 days; bottom, cells subjected to ethanol withdrawal for 6 h. Magnification, 50×.

GHB also fails to modulate the GABA-evoked Cl^- currents in cerebellar granule cells and hippocampal pyramidal neurons in culture (E. Sanna, unpublished observations).

Discussion

We have shown that long-term exposure of primary rat cerebellar granule cells to a high concentration (100 mM) of ethanol results in a decrease in the abundance of $GABA_AR$ γ_2 subunit mRNAs, consistent with the previous observation that prolonged alcohol administration induces a reduction in GABAergic transmission in rat brain (Sanna et al., 1993). This effect of long-term ethanol treatment in cultured cerebellar granule cells is similar to that of long-term treatment with benzodiazepines or neurosteroids in the same culture system (Follesa et al., 2000, 2001). Long-term ethanol treatment did not affect the abundance of α_1, α_4, or α_6 subunit mRNAs in the cultured neurons.

In an attempt to characterize the mechanism responsible

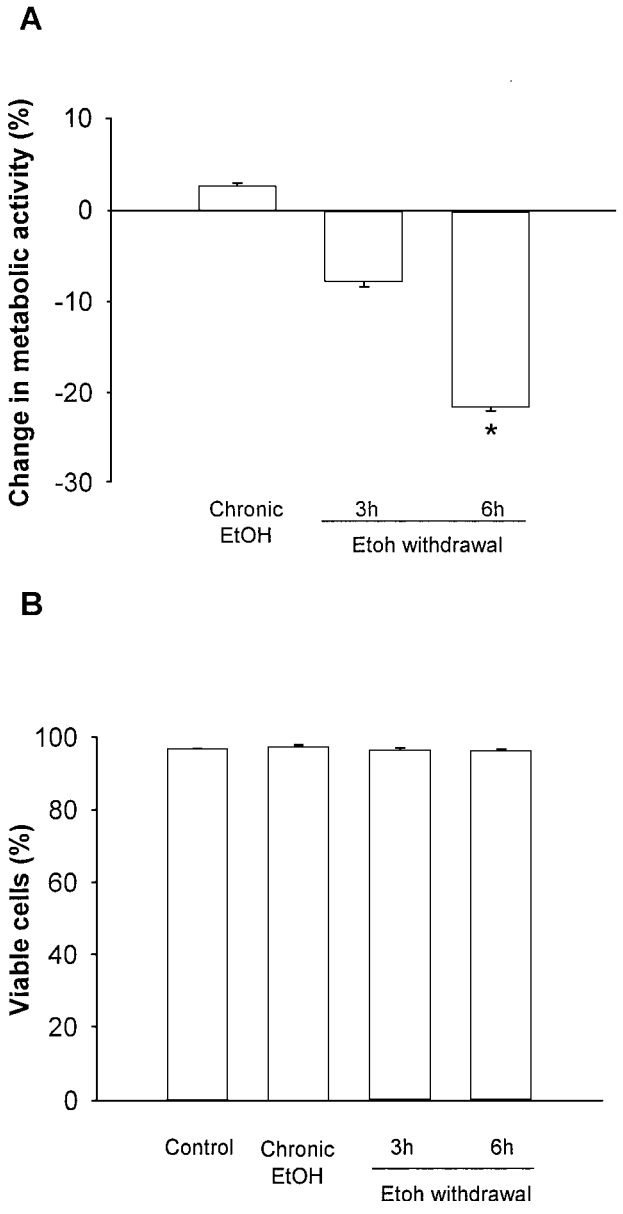

Fig. 5. Effects of chronic treatment with and withdrawal of ethanol on the metabolic activity (A) and viability (B) of cerebellar granule cells. A, cells were treated with 100 mM ethanol for 5 days (chronic EtOH) and then subjected to ethanol withdrawal for 3 or 6 h. Cellular metabolic activity was assessed by the spectrophotometric determination of resazurin reduction. Data are expressed as percentage change in metabolic activity relative to the metabolic activity of control cells not exposed to ethanol and are means ± S.E.M. of values from three independent experiments. *, $p < 0.05$ versus control (ANOVA and Scheffe's test). B, after measurement of metabolic activity, the cells were harvested by exposure to trypsin, and the number of viable cells was counted with a hemocytometer after staining with trypan blue. Data are means ± S.E.M. of three independent experiments.

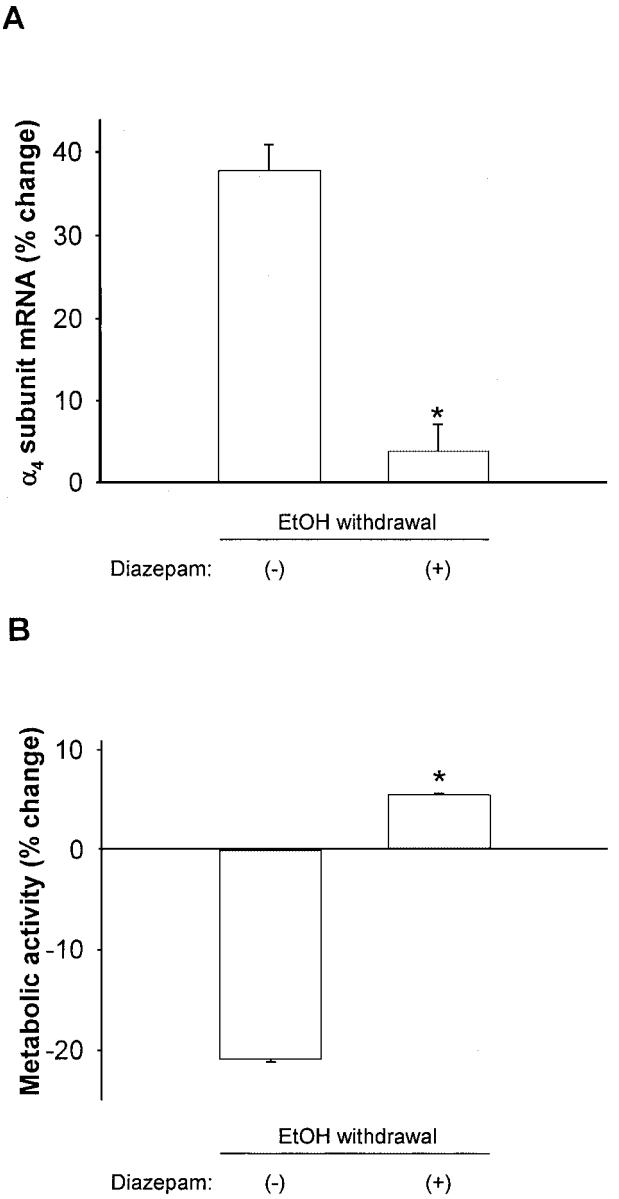

Fig. 6. Antagonism by diazepam both of the increase in the abundance of the $GABA_AR$ α_4 subunit mRNA (A) and of the impairment in cellular metabolism (B) induced by ethanol withdrawal in cerebellar granule cells. Cells were incubated first for 5 days with 100 mM ethanol and then for 3 h (A) or 6 h (B) after ethanol withdrawal in the absence or presence of 10 μM diazepam. The abundance of the α_4 subunit mRNA was measured by RNase protection assay (A) and cellular metabolic activity was measured with resazurin (B). Data are means ± S.E.M. of values from three independent experiments and are expressed as percentage change relative to the values for control cultures not exposed to ethanol. *, $p < 0.01$ versus ethanol withdrawal without diazepam (ANOVA and Scheffé's F test).

for the development of ethanol dependence, several laboratories have previously examined the effects of ethanol on the abundance of $GABA_AR$ subunit mRNAs and peptides in the brain, obtaining different results that seem to depend on the method and time of intoxication used or the brain region examined (for review, see Grobin et al., 1998). Ethanol administration for 12 weeks has thus previously been shown to induce a decrease in the abundance of the α_1 subunit mRNA and peptide in the rat hippocampus (Charlton et al., 1997); such treatment for shorter time (2 weeks), however, had no effect in hippocampus, cerebellum, and frontal cortex (Charlton et al., 1997). Other studies, on the contrary, show that the amount of the α_1 subunit itself was reduced in the cerebral cortex and cerebellum by long-term treatment of rats with ethanol, whereas in the cerebellum, the α_6 subunit was increased (for review, see Grobin et al., 1998). These last observations were not fully supported by binding studies; in fact, an increase or no change in [^{3}H]zolpidem binding was observed in the same brain areas (for review, see Grobin et al., 1998). With such a decrease in the expression of the α_1 mRNA subunit and peptide, however, one should expect a decrease in [^{3}H]zolpidem binding. A more recent study (Mehta and Ticku, 1999b), in agreement with our present data, demonstrated that long-term ethanol administration

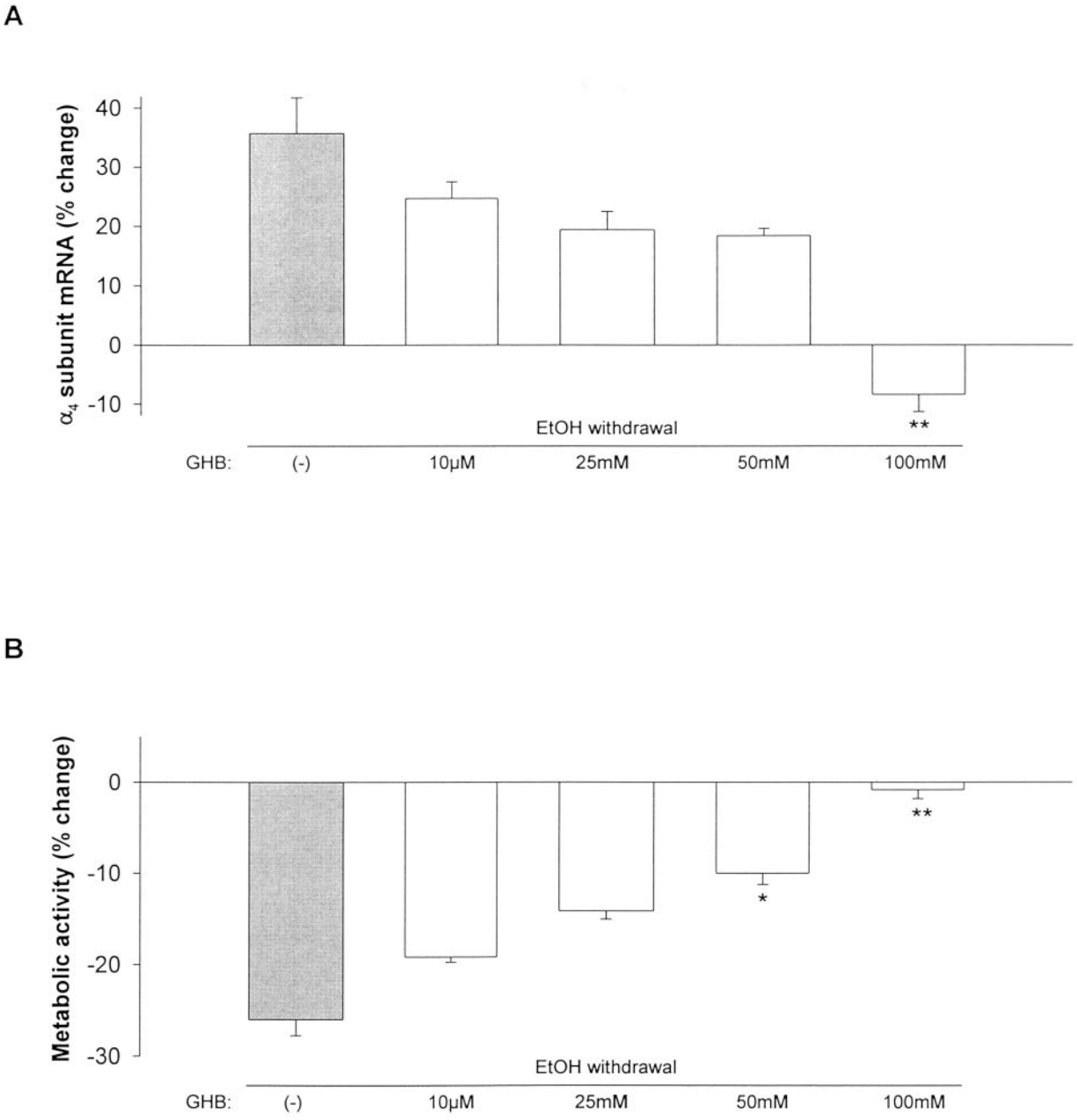

Fig. 7. Dose-dependent antagonism by GHB both of the increase in the abundance of the $GABA_AR$ α_4 subunit mRNA (A) and of the impairment in cellular metabolism (B) induced by ethanol withdrawal in cerebellar granule cells. Cells were incubated first for 5 days with 100 mM ethanol and then for 3 h (A) or 6 h (B) after ethanol withdrawal in the absence or presence of the indicated concentrations of GHB. The abundance of the α_4 subunit mRNA was measured by RNase protection assay (A) and cellular metabolic activity was measured with resazurin (B). Data are means ± S.E.M. of values from seven (A) or three (B) independent experiments and are expressed as percentage change relative to the values for control cultures not exposed to ethanol. *, $p < 0.05$; **, $p < 0.01$ versus ethanol withdrawal without GHB (ANOVA and Scheffé's F test).

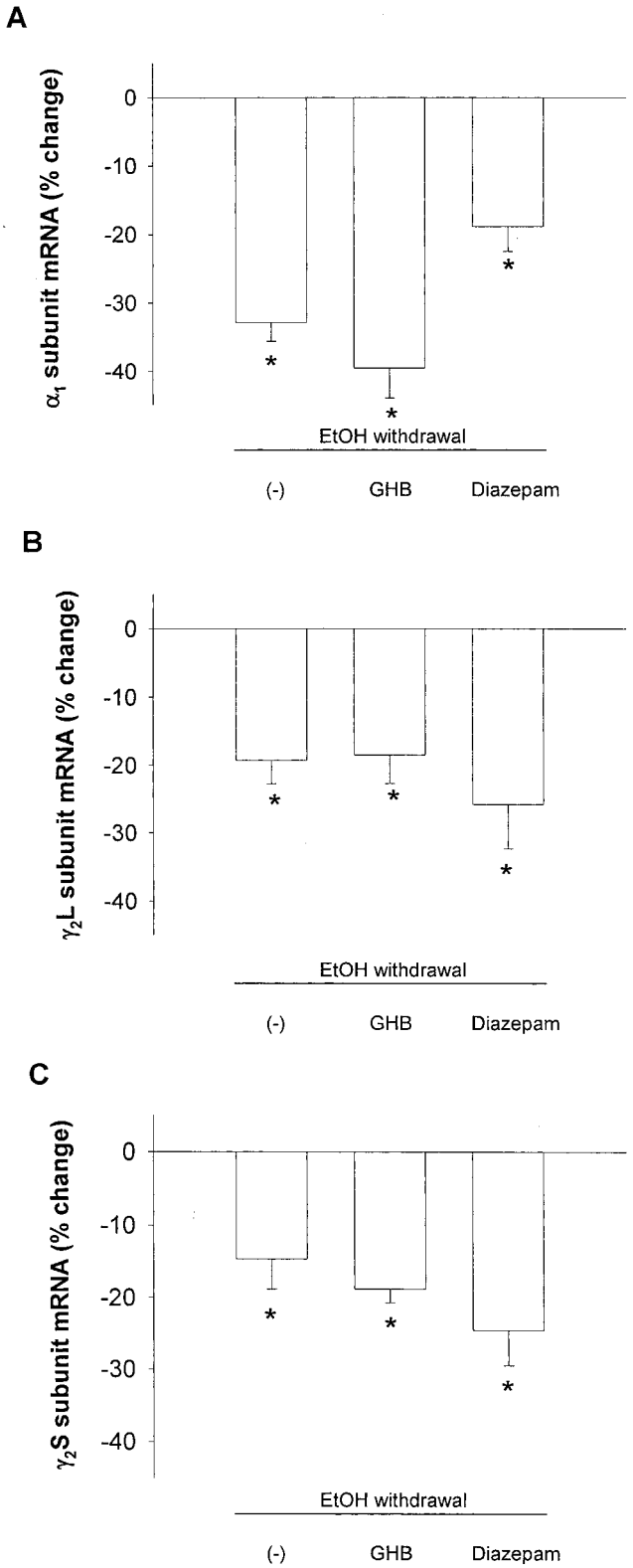

Fig. 8. Lack of effect of GHB or diazepam on the decrease in the abundance of $GABA_AR$ α_1 (A), γ_2L (B), and γ_2S (C) subunit mRNAs associated with ethanol withdrawal in cerebellar granule cells. Cells were incubated first for 5 days with 100 mM ethanol and then for 3 h after ethanol withdrawal in the absence or presence of 100 mM GHB or 10 μM diazepam. The amounts of $GABA_AR$ mRNAs were determined by RNase protection assay. Data are means ± S.E.M. of values from three independent experiments and are expressed as percentage change relative to the values for control cultures not exposed to ethanol. *, $p < 0.01$ versus control (ANOVA and Scheffé's F test).

did not result in down-regulation of $GABA_AR$ assemblies containing the α_1 subunit in the rat cerebral cortex or cerebellum, as determined by labeling of the receptors with [^{3}H]muscimol, [^{3}H]flunitrazepam, [^{3}H]Ro 15-4513, or [^{3}H]zolpidem and immunoprecipitation with antibodies specific for the α_1 subunit. This last observation, although consistent with our data, is in disagreement with a previous study from the same group, in which long-term ethanol treatment increased the [^{3}H]Ro 15-4513 binding (Mhatre et al., 1988).

Given that the genes encoding for the α_1 and α_6 subunits are localized in the same cluster, the direction of change in their gene expression should be the same, because these genes are coregulated (Holt et al., 1996). Our results in cerebellar granule cells in culture are consistent with the above hypothesis showing a similar patterns of expression of the α_1 and α_6 genes in cells subjected either to long-term ethanol treatment (no change in abundance) or to ethanol withdrawal (a decrease in abundance). Thus, the apparent discrepancies in the effects of long-term ethanol exposure on the gene expression of these two subunits might be attributable to the difficulty in optimizing, in the studies "in vivo," the timing between consecutive ethanol administrations so as to prevent the onset of withdrawal effects. On the contrary, in our model system, it is very simple to perform ethanol withdrawal. Thus, the abrupt discontinuation of ethanol treatment resulted in a decrease in the abundance of the α_1 and α_6 subunit mRNAs, as well as prolongation and enhancement of the decrease in the amounts of both γ_2 subunit mRNAs. Ethanol withdrawal also induced a marked increase in the abundance of the α_4 subunit mRNA. This latter effect was rapid and therefore might be important in the onset of withdrawal syndrome. These changes in $GABA_AR$ gene expression are identical to those induced by withdrawal of either benzodiazepines (Follesa et al., 2001), imidazopyridines and pyrazolopyrimidines (Follesa et al., 2002), or neurosteroids (Follesa et al., 2000). These molecular changes thus might reflect a common mechanism by which diazepam and ethanol trigger changes in receptor function that in vivo might account for the development of withdrawal symptoms. The presence of the α_4 subunit in recombinant $GABA_AR$ is associated with a reduced sensitivity to classical benzodiazepine agonists and to zolpidem as well as with a distinct pattern of regulation (positive rather than no allosteric modulation) by flumazenil. The patch-clamp studies demonstrated that in the ethanol-withdrawn granule cells, flumazenil positively modulates the $GABA_AR$ function, in agreement with the observation that in these cells, ethanol withdrawal produced up-regulation of the α_4 subunit mRNA. Thus, the increase in the abundance of the α_4 subunit mRNA induced by withdrawal of ethanol, diazepam, or neuroactive steroids might contribute to changes in the sensitivity of $GABA_AR$ to drugs and endogenous modulators.

The effects of ethanol withdrawal on $GABA_AR$ gene expression were accompanied by a decrease in cellular metabolic activity. This impairment in metabolism also might play a role in the development of dependence on ethanol or it might represent a homeostatic response of the neurons to the sudden lack of ethanol in the culture medium. Long-term exposure to ethanol (100 mM) had no apparent effect on metabolic activity or on neuronal morphology and was not cytotoxic, given that the number of viable cells was unchanged.

Benzodiazepines are one of the best treatments available for the life-threatening condition of alcohol withdrawal syndrome in humans (Mayo-Smith, 1997). These drugs prevent the more severe clinical manifestations of the syndrome, such as seizures and delirium. GHB has also more recently been proposed as an alternative treatment to reduce alcohol consumption and craving in persons with alcoholism. In laboratory animals, GHB and alcohol exhibit cross-tolerance to their mutual side effects (Colombo et al., 1995). Moreover, GHB reduces self-administration of alcohol and suppresses alcohol withdrawal signs in alcohol-preferring rats (Fadda et al., 1989). A comparison between benzodiazepines and GHB in the management of alcohol withdrawal syndrome in humans revealed that GHB is as effective as diazepam and seems to reduce anxiety, agitation, and depression more rapidly (Addolorato et al., 1999).

The substitution of diazepam for ethanol after long-term ethanol treatment completely antagonized the marked increase in the abundance of the $GABA_AR$ α_4 subunit mRNA, the decrease in cellular metabolic activity induced by ethanol withdrawal, and the flumazenil potentiation. The substitution of very high GHB concentrations for ethanol was as effective as diazepam in antagonizing the same effects. In contrast, neither diazepam nor GHB had any effect on the changes in the abundance of the α_1 and γ_2 subunit mRNAs observed during ethanol withdrawal. Given that the down-regulation of both γ_2 subunits was trigged by long-term ethanol treatment, it is not surprising that diazepam did not antagonize this effect. On the other hand, it is more difficult to explain the lack of effect on the α_1 subunit. Nevertheless, we can hypothesize that higher concentrations of diazepam might be necessary to overcome the decrease of the α_1 subunit induced by ethanol withdrawal. Because the α_1 subunit has been demonstrated to mediate the sedative-hypnotic effects of benzodiazepines (Rudolph et al., 1999), our speculation is supported by the clinical observation that to sedate patients during alcohol withdrawal massive doses and con-

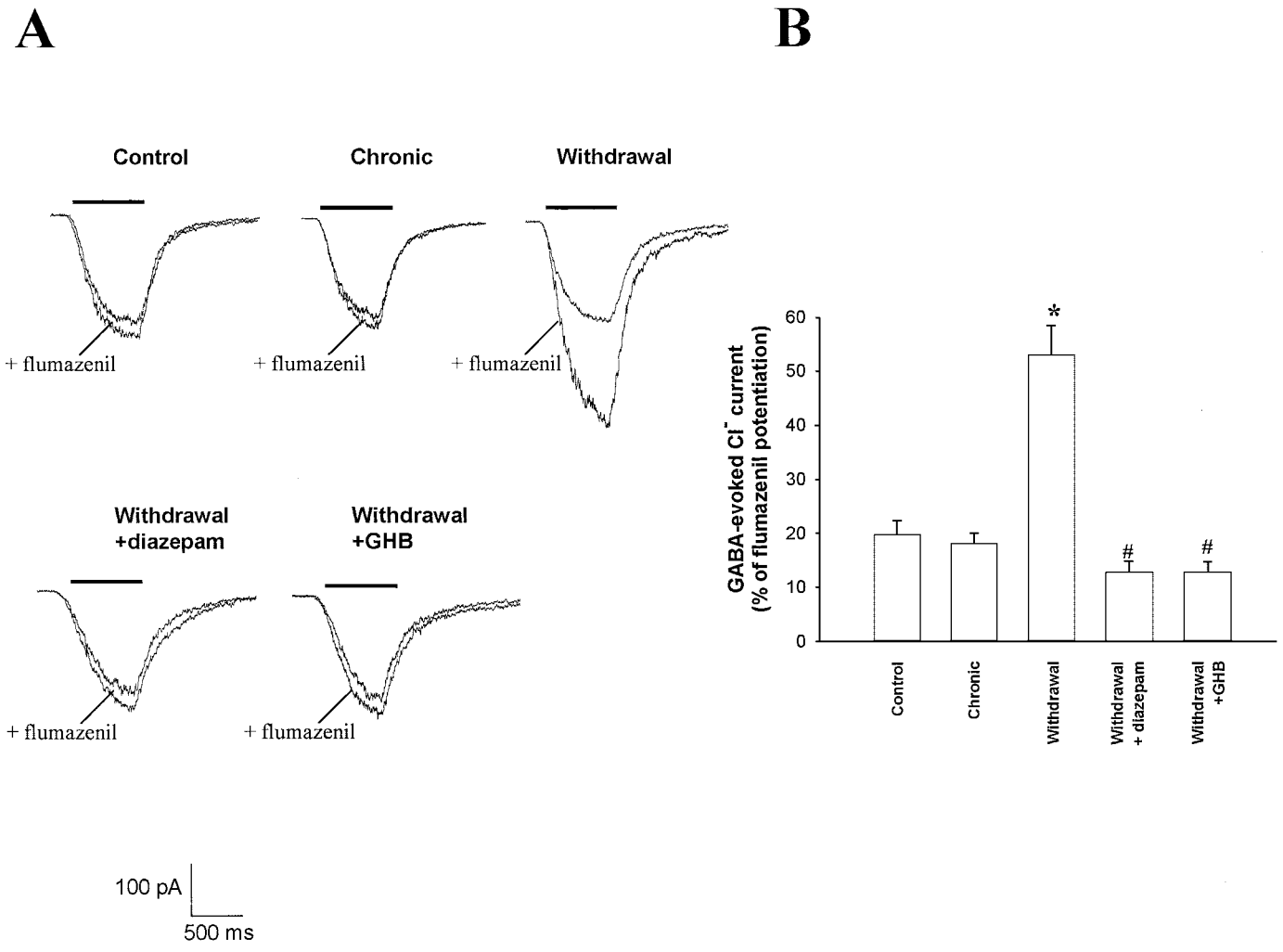

Fig. 9. Potentiation of $GABA_AR$ function by flumazenil in ethanol withdrawn cerebellar granule cells: reversal by diazepam and GHB. Cells were treated with 100 mM ethanol for 5 days (chronic) or subjected to ethanol withdrawal for 6 h (withdrawal). In other two groups of cells, 10 μM diazepam (withdrawal+diazepam) or 100 mM GHB (withdrawal+GHB) were substituted for ethanol for 6h. Whole-cell patch-clamp electrophysiological recording was performed by applying GABA at a concentration of 1 to 3 μM, which induced a current with an amplitude of 5 to 10% of the maximal response (EC_{5-10}). GABA was then coapplied with flumazenil 3 μM. A, representative electrophysiological recording traces of the indicated experimental groups. B, means ± S.E.M. values of 6 (control and chronic) to 7 (withdrawal, withdrawal+diazepam, withdrawal+GHB) recordings from individual neurons expressed as percentage of flumazenil potentiation of the response to GABA. *, $P < 0.01$ versus control; #, $P < 0.01$ versus withdrawal.

tinuous infusion of benzodiazepines are necessary (Sellers et al., 1983).

The antagonism by diazepam and very high concentrations of GHB on the changes in α_4 subunit mRNA, consequent receptor function, and cellular metabolism induced by ethanol withdrawal support the possible crucial role of the α_4 subunit in the molecular mechanisms of withdrawal. Withdrawal from steroids has previously been shown to alter the kinetics of $GABA_AR$-mediated currents in the rat hippocampus as well as to increase both the abundance of the α_4 subunit and anxiety in pseudopregnant animals (Smith et al., 1998b). Furthermore, suppression of the increase in the abundance of the α_4 subunit prevents withdrawal signs associated with endogenous steroids in a progesterone withdrawal paradigm (Smith et al., 1998a).

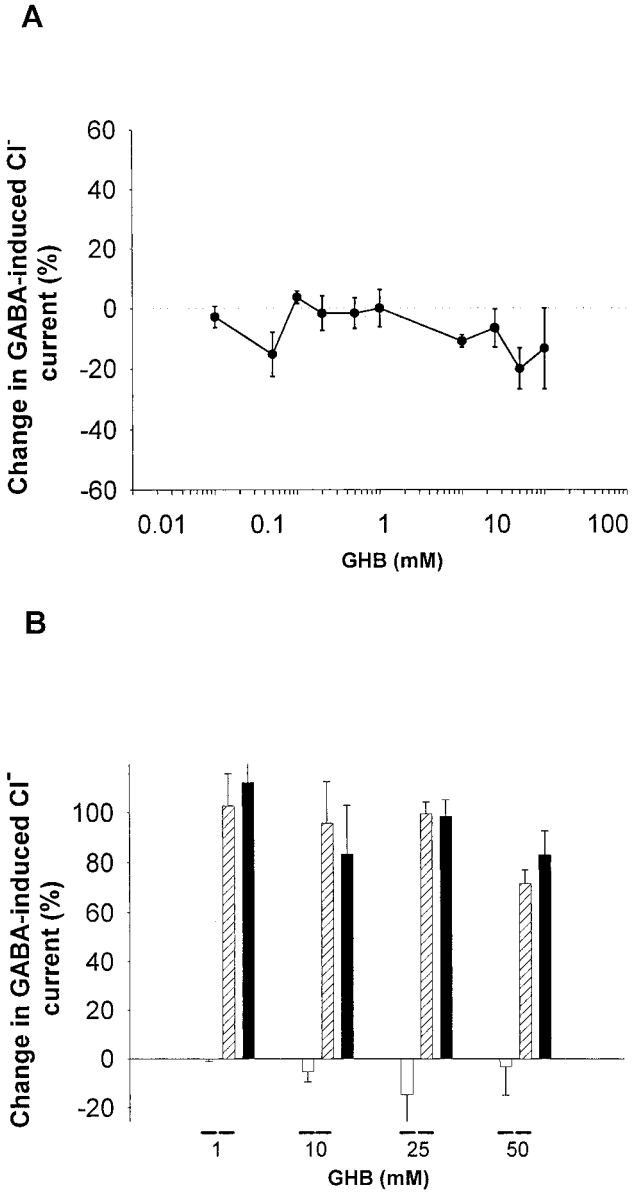

Fig. 10. Lack of effect of GHB on the function of recombinant $GABA_AR$ ($\alpha_{1\beta2\gamma2}L$) expressed in *X. laevis* oocytes. A, oocytes were exposed to the indicated concentrations of GHB for 1 min before coapplication with GABA at an $EC_{5\text{ to }10}$ (6 to 10 μM) for 30 s. B, oocytes were exposed to GHB alone at the indicated concentrations (open bars), to GHB plus 1 μM diazepam (striped bars), or to 1 μM diazepam alone (solid bars) for 1 min before respective coapplication with GABA at an $EC_{5\text{ to }10}$ for 30 s. Data in A and B are expressed as percentage change of the GABA-induced Cl^- current and are means ± S.E.M. of values from four oocytes.

Whereas the antagonism by diazepam we observed is consistent with the specific action of this drug at the $GABA_AR$ complex, the mechanism by which GHB induces this same effect is not clear. Consistent with previous data showing that GHB does not seem to possess affinity for [^{3}H]muscimol, *t*-[^{35}S]butylbicyclophosphorothionate, or [^{3}H]flunitrazepam binding sites (Serra et al., 1991; Bernasconi et al., 1992), our present results on the $GABA_AR$ function indicate that GHB does not directly affect the activity of the $GABA_AR$ nor does it affect the action of allosteric modulators such as benzodiazepines. The affinities of GHB for its own specific receptor and for the $GABA_B$ receptor are in the nanomolar and micromolar ranges, respectively (Bernasconi et al., 1992). Thus, despite the similarities between the pharmacological properties of GHB and those of sedative-hypnotic drugs, the effects of GHB do not seem to be mediated by $GABA_AR$. Rather, most of the effects of GHB, especially those induced by high concentrations of this drug, seem to be mediated by $GABA_B$ receptors or to be nonspecific. Thus, both biochemical and behavioral effects of high doses of GHB are reproduced or potentiated by $GABA_B$ receptor agonists (Bernasconi et al., 1999). Moreover, like GHB, the $GABA_B$ receptor agonist baclofen also protects against alcohol dependence (Bernasconi et al., 1999).

In our experimental paradigm, the antagonistic action elicited by GHB on the ethanol withdrawal-induced up-regulation of the α_4 subunit of the $GABA_AR$, receptor function, and cellular metabolism were observed only at the concentration of 100 mM. This concentration of GHB was very high compared with that hypothetically achieved in human studies (Addolorato et al., 1999). The physiological concentration of GHB in the mammalian brain ranges from 2 to 5 μM, but this amount could be increased by several orders of magnitude after exogenous administration of GHB (Gobaille et al., 1999). Thus, we can speculate that the antagonistic action of GHB in vivo could be the result of a synergic interaction between the elevated concentration of GHB and other endogenous modulators of neurotransmission. Accordingly, both ethanol and GHB greatly increase the levels of $GABA_AR$ active steroids in the rat brain (Morrow et al., 2001; Barbaccia et al., 2002). Thus, in vivo, a lower dose of GHB might be sufficient to antagonize the effects of ethanol. The same might not hold be true in neurons in culture, where the concentrations of steroids (Follesa et al., 2000) are irrelevant.

In conclusion, our data demonstrate that the ethanol withdrawal-induced increase in the expression of the $GABA_AR$ α_4 subunit gene in cultured rat cerebellar granule cells is prevented by diazepam and very high concentrations (100 mM) of GHB, two of the most widely used drugs in the treatment of alcohol withdrawal syndrome in humans. This action of GHB does not seem to be mediated by specific activation of $GABA_AR$. A rapid and marked increase in the abundance of the α_4 subunit may thus contribute to the development of alcohol withdrawal symptoms that are ameliorated by both GHB and diazepam.

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