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Inhibition by miltirone of up-regulation of $GABA_A$ receptor α_4 subunit mRNA by ethanol withdrawal in hippocampal neurons

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Abstract

Miltirone, a tanshinone isolated from the root of *Salvia miltiorrhiza*, has been characterized as a low-affinity ligand for central benzodiazepine receptors. We have now shown that this compound bound with low affinity (micromolar range) to central benzodiazepine recognition sites but did not interact with peripheral benzodiazepine receptors. It failed to potentiate Cl^- currents induced by γ-aminobutyric acid (GABA) both in *Xenopus* oocytes expressing recombinant human $GABA_A$ receptors and in cultured rat hippocampal pyramidal cells, but it inhibited the ability of diazepam to potentiate the effect of GABA in these systems. Miltirone (1–10 μM) also partially inhibited the increase in the abundance of the mRNA for the α_4 subunit of the $GABA_A$ receptor induced by ethanol withdrawal in cultured hippocampal neurons. These results suggest that miltirone might ameliorate the symptoms associated with discontinuation of long-term administration of ethanol or of other positive modulators of the $GABA_A$ receptor.

Keywords: Miltirone; $GABA_A$ receptor; Ethanol withdrawal; Dependence

1. Introduction

γ-Aminobutyric acid type A ($GABA_A$) receptors play an important role in the central effects of ethanol, in particular in the development both of dependence during long-term ethanol administration and of withdrawal symptoms after its discontinuation (Aguayo et al., 2002; Grobin et al., 1998; Suwaki et al., 2001). Acute or chronic ethanol exposure thus modifies the function of these receptors as well as the expression of receptor subunit genes both in vitro and in vivo (Devaud et al., 1996, 1997; Follesa et al., 2003; Mehta and Ticku, 1999). Consistent with the role of $GABA_A$ receptors in the neuropharmacology of ethanol, benzodiazepines are among the most widely used drugs for the clinical treatment of ethanol dependence and withdrawal syndrome (Lejoyeux et al., 1998).

γ-Hydroxybutyric acid, a normal constituent of the mammalian brain that exerts hypnotic and anesthetic effects when administered in pharmacological doses, also ameliorates ethanol withdrawal syndrome in both rats (Fadda et al., 1989) and humans (Addolorato et al., 2002) with an efficacy similar to that of benzodiazepines. Although this compound does not directly interact with $GABA_A$ receptors (Follesa et al., 2003; Serra et al., 1991), we have recently shown that, like diazepam, it prevents changes in the abundance of various $GABA_A$ receptor subunit mRNAs elicited by ethanol withdrawal in hippocampal and cerebellar cells in culture (Follesa et al., 2003; Sanna et al., in press). Together, these observations suggest that the ability to inhibit changes in $GABA_A$ receptor gene expression is a common feature of certain drugs that prevent or reduce the symptoms of ethanol withdrawal syndrome, albeit with otherwise different mechanisms of action.

Miltirone is a compound present in the root of the Chinese medicinal herb *Salvia miltiorrhiza* and acts as a low-affinity ligand at benzodiazepine receptors (Chang et al., 1991; Lee et al., 1991). It increases the duration of the

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hypnotic effects of chloral hydrate and barbiturates in mice. This compound, which has been characterized as a partial agonist of the central benzodiazepine receptor, has been suggested for use as a tranquilizer devoid of the sedative and addictive effects of classical benzodiazepines (Chang et al., 1991; Lee et al., 1991). To characterize further the mechanism of action of miltirone as well as its potency and intrinsic activity at $GABA_A$ receptors, we have now evaluated its effects both at the molecular level and electrophysiologically. Moreover, we have examined whether miltirone, like benzodiazepines (Follesa et al., 2003; Sanna et al., in press), is able to inhibit the changes in $GABA_A$ receptor gene expression elicited by ethanol withdrawal in cultured hippocampal cells.

2. Materials and methods

2.1. Drugs

Miltirone was obtained from Indena (Milan, Italy). Diazepam was kindly provided by Ditta FIS-Alte Montecchio (Vicenza, Italy).

2.2. Animals

Male Sprague–Dawley rats were obtained from Charles River (Como, Italy). Animals were housed six per cage under an artificial 12-h light, 12-h dark cycle (light on from 0800 to 2000 h) at a constant temperature of 22 ± 2 °C and a relative humidity of 65%. They had free access to water and standard laboratory food. Animal care and handling were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocols were also approved by the Animal Ethics Committee of the University of Cagliari.

2.3. [^{3}H]Flunitrazepam binding assay

Rats were killed with a guillotine, and the cerebral cortex was rapidly dissected from the brain and homogenized with a Polytron PT 10 (setting 5, 20 s) in 10 volumes of ice-cold 50 mM Tris–HCl (pH 7.4 at 25 °C). The homogenate was centrifuged at $39,000 \times g$ for 10 min, and the resulting pellet was resuspended in 50 volumes of 50 mM Tris–HCl buffer and used for the assay. [^{3}H]flunitrazepam binding was determined in a final volume of 1000 μl, consisting of 400 μl of cerebral cortical membranes (0.4–0.5 mg of protein), 100 μl of [^{3}H]flunitrazepam (70–100 Ci/mmol; final assay concentration, 0.5 nM), 5 μl of test drug or vehicle (dimethyl sulfoxide), and 495 μl of 50 mM Tris–HCl buffer. Incubations (at 0 °C) were initiated by the addition of membranes and were terminated after 60 min by rapid filtration through glass-fiber strips (Whatman GF/B) in a Cell Harvester filtration manifold (model M-24, Brandel). The filters were washed with two 4-ml portions of ice-cold 50 mM Tris–HCl buffer, after which filter-bound radioactivity was quantified by liquid scintillation spectrometry. Nonspecific binding was defined as binding in the presence of 5 μM diazepam.

2.4. [^{3}H]PK11195 binding

Rats were killed with a guillotine, and the cerebral cortex was rapidly dissected from the brain and stored at −80 °C. On the day of the experiment, the tissue was homogenized with a Polytron PT 10 (setting 5, 20 s) in 50 volumes of ice-cold modified Dulbecco's phosphate-buffered saline (PBS) (pH 7.4 at 25 °C). The homogenate was centrifuged at $39,000 \times g$ for 30 min, and the resulting pellet was resuspended in 50 volumes of PBS and again centrifuged at $39,000 \times g$ for 30 min. The new pellet was resuspended in 10 volumes of PBS and used for the assay. 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide (PK11195) [^{3}H]PK11195 binding was determined in a final volume of 1000 μl, consisting of 100 μl of cerebral cortical membranes (0.15–0.20 mg of protein), 100 μl of [^{3}H]PK11195 (80–100 Ci/mmol; final assay concentration, 1 nM), 5 μl of test drug or vehicle and 795 μl of PBS. Incubations (25 °C) were initiated by the addition of membranes and were terminated after 90 min by rapid filtration through glass-fiber strips (Whatman GF/B). The filters were washed with two 4-ml portions of ice-cold PBS and filter-bound radioactivity was quantified by liquid scintillation spectrometry. Nonspecific binding was defined as binding in the presence of 10 μM PK11195.

2.5. Microinjection of cDNAs into Xenopus oocytes and electrophysiological recording

Xenopus laevis oocytes (stage V to VI) were isolated as described previously (Lin et al., 1992). The cDNAs for the α_1, α_2, β_2, and γ_2L subunits of the human $GABA_A$ receptor were subcloned into the pCDM8 vector (Invitrogen, San Diego, CA) A mixture of plasmids encoding either α_1, β_2, and γ_2L or α_2, β_2, and γ_2L receptor subunits (total of 1.5 ng of cDNA in 30 nl in a 1:1:1 ratio) was injected into the nucleus of oocytes as described (Colman, 1984). Electrophysiological measurements were performed on oocytes 1–4 days after injection. Oocytes expressing $\alpha_1\beta_2\gamma_2$L or $\alpha_2\beta_2\gamma_2$L receptors were placed in a chamber (capacity, ~ 100 μl) and perfused (2 ml/min) with Modified Barth's solution (MBS): [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 $Ca(NO_3)_2$, 0.91 mM $CaCl_2$] with the use of a roller pump (Cole-Parmer Instruments, Chicago, IL). The animal pole of each oocyte was impaled with two glass electrodes (0.5 to 10 MΩ) filled with 3 M KCl, and the cells were subjected to voltage clamp at −70 mV (oocyte clamp OC-725 C; Warner Instruments, Ham-

den, CT). Oocytes were exposed for 30 s to GABA dissolved in MBS. Miltirone and diazepam were first dissolved in dimethyl sulfoxide and then diluited in MBS; the final concentration of solvent to which the oocytes were exposed was 1% and did not affect the response to GABA. Miltirone (or diazepam) was applied for 60 s alone before coapplication with GABA for 30 s. Oocytes were exposed to MBS alone for 5 or 20 min between applications of test drugs at concentrations of ≤ 0.3 or >0.3 μM, respectively.

2.6. Primary culture of hippocampal neurons

Primary cultures of hippocampal neurons were prepared from rats on postnatal days 1 to 3 as described previously (Costa et al., 2000), with minor modifications. Pups were killed by decapitation, and the hippocampus was removed and transferred to a culture dish containing Neurobasal A medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 25 μM glutamate, 0.5 mM glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml). The tissue was chopped with scissors, transferred to a sterile tube, and gently dissociated by repeated passage through a Pasteur pipette with an opening of 0.5 mm. The dissociated cells were plated either in 35-mm culture dishes (4×10^6 cells) that had been coated with poly-L-lysine hydrobromide (100 μg/ml; 30–70 kDa) (Sigma) for measurement of $GABA_A$ receptor α_4 subunit mRNA or in multiwell dishes containing 12-mm round glass cover slips (6×10^5 cells) that had been coated with poly-L-lysine for electrophysiological recording. Cells were cultured in a humidified incubator containing 5% CO_2 at 37 °C. Twenty-four hours after plating, fetal bovine serum was replaced by B-27 supplement (Invitrogen), and glutamate was removed from the medium after 3 days of culture.

After 5 days in culture, cells were exposed continuously for the next 6 days to 100 mM ethanol, with replacement of the medium every 24 h. In withdrawal experiments, the ethanol-containing medium was replaced after 6 days by ethanol-free medium containing (or not) miltirone (1 or 10 μM) or diazepam (10 μM). Ethanol was dissolved in medium, whereas miltirone and diazepam were dissolved in dimethyl sulfoxide and subsequently diluted to the desired concentration in culture medium. Control neurons were treated with the corresponding vehicle.

2.7. Whole-cell electrophysiological recording

Immediately before recording, coverslips containing hippocampal neurons were transferred to a perfusion chamber (Warner Instruments) and the cells were observed with a Nikon upright microscope equipped with Nomarski optics (40 ×). Large neurons with a pyramidal shape and well-defined dendritic processes were selected for electrophysiological recording. The membrane potential was clamped at −60 mV with an Axopatch 200-B amplifier (Axon Instruments, Foster City, CA). The resting membrane potential for the studied neurons was about −60 mV. Recording pipettes (borosilicate capillaries with a 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. Currents through the patch-clamp amplifier were filtered at 2 kHz and digitized at 5.5 kHz with 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–NaOH (pH 7.3), and 11 mM glucose. The internal solution comprised 140 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES–CsOH (pH 7.3), and 2 mM ATP (disodium salt) with a fast-exchange flow-tube perfusion system driven by a motor (Warner Instruments). All experiments were performed at room temperature (23–25 °C). Data were analyzed with pClampfit 8.01 software (Axon Instruments). Modulation of GABA-evoked Cl^- currents by test drugs was expressed as percentage change, $[(I'/I) - 1]100\%$, where I is the average of control responses to GABA obtained before drug application and after drug washout, and I' is the average of the GABA-induced responses obtained from the same cell in the presence of drug.

2.8. Riboprobe preparation

The cDNA for the α_4 subunit of the $GABA_A$ receptor was prepared as described (Follesa et al., 1998) by reverse transcription and the polymerase chain reaction. In brief, cDNA prepared from rat brain (1–10 ng) was subjected to amplification with *Taq* DNA polymerase (2.5 U) (Perkin-Elmer/Cetus, Norwalk, CT) in 100 μl of standard buffer [100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin] containing 1 μM each of specific sense and antisense primers and 200 μM of each deoxynucleoside triphosphate. The primers were designed to include cDNA sequences with the lowest degree of homology among the different receptor subunits. The reaction was performed in a thermal cycler for 30 cycles of incubation at 94 °C for 45 s and 60 °C for 1 min, with a final extension at 72 °C for 15 min. The reaction product was separated by electrophoresis, visualized by staining with ethidium bromide, excised from the gel, purified, and cloned into the pAMP 1 vector (Invitrogen). The resulting plasmid was introduced into *Escherichia coli* DH5α and subsequently purified from the bacterial cells, and the cDNA insert was sequenced with a Sequenase DNA sequencing kit (USB, Cleveland, OH). The determined nucleotide sequence was 100% identical to the previously published sequence. The plasmid containing the α_4 subunit cDNA was linearized with *Hin*dIII and used as a template for SP6 RNA polymerase to generate an

[α-^{32}P]UTP-labeled cRNA probe for an RNase protection assay.

2.9. RNA extraction and measurement of α_4 subunit mRNA

Total RNA was isolated from cultured hippocampal cells with an RTN kit (Sigma) and quantified by measurement of absorbance at 260 nm. An RNase protection assay for the semiquantitative measurement of the mRNA for the α_4 subunit of the $GABA_A$ receptor was performed as described (Follesa et al., 1998). In brief, 15 μg of total RNA were dissolved in 20 μl of hybridization solution containing 150,000 cpm of ^{32}P-labeled cRNA probe for the α_4 subunit mRNA (6×10^7 to 7×10^7 cpm/μg) and 15,000 cpm of ^{32}P-labeled cyclophilin cRNA (1×10^6 cpm/μg). (Cyclophilin is expressed widely among tissues, including the brain, and its gene is most likely regulated in an "on or off" manner; cyclophilin mRNA was thus used as an internal standard for our measurements.) 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 the α_4 subunit and cyclophilin mRNAs were determined by measurement of the optical density of the corresponding bands on the autoradiogram with a densitometer (model GS-700; BioRad, Hercules, CA), which was calibrated to detect saturated values so that all measurements were in the linear range. The data were normalized by dividing the optical density of the protected fragment for the α_4 subunit mRNA by that of the protected fragment for cyclophilin mRNA. The amount of α_4 subunit mRNA was therefore expressed in arbitrary units.

2.10. Statistical analysis

Data are presented as means ± S.E.M. The statistical significance of differences was assessed by analysis of variance followed by Scheffe's test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Effects of miltirone on [^{3}H]flunitrazepam and [^{3}H]PK11195 binding to cortical membranes

The ability of miltirone to interact with central benzodiazepine receptors was investigated by examining its effect on the binding of [^{3}H]flunitrazepam to rat cerebrocortical membranes. The binding of [^{3}H]flunitrazepam was inhibited by miltirone in a dose-dependent manner, with a IC_{50} of 1.36 ± 0.12 μM (Fig. 1). In the same membrane preparation, diazepam inhibited [^{3}H]flunitrazepam binding with a potency (IC_{50}) of 11.5 ± 0.1 nM higher than that of miltirone. In contrast, miltirone (10 μM) failed to interact with the peripheral benzodiazepine receptor, as revealed by its lack of effect on [^{3}H]PK11195 binding to rat cerebrocortical membranes (Fig. 1, insert).

3.2. Effects of miltirone on recombinant $GABA_A$ receptors expressed in Xenopus oocytes

The effects of miltirone (0.1–30 μM) on $GABA_A$ receptor function were assessed with human recombinant $\alpha_1\beta_2\gamma_2L$ and $\alpha_2\beta_2\gamma_2L$ receptors expressed in *Xenopus*

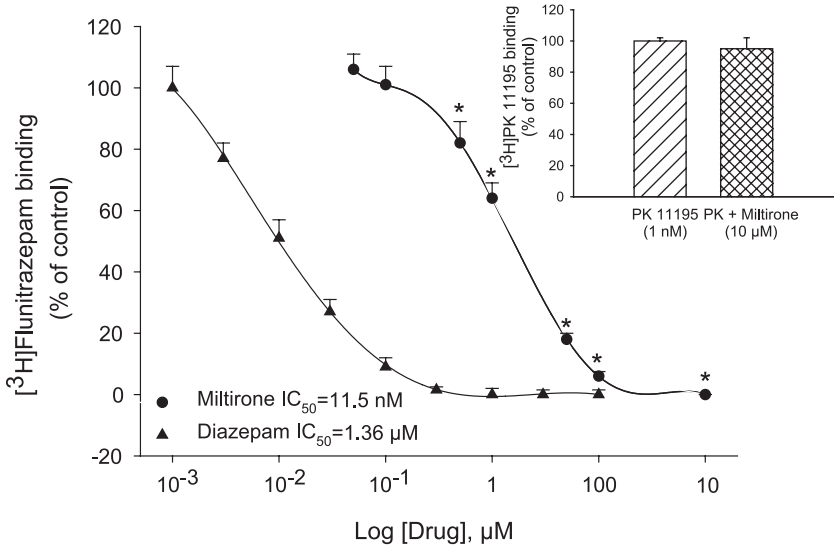

Fig. 1. Effects of miltirone or diazepam on the binding of [^{3}H]flunitrazepam or [^{3}H]PK11195 to rat cerebrocortical membranes. Membranes were incubated with 0.5 nM [^{3}H]flunitrazepam or 1 nM [^{3}H]PK11195 (insert) in the presence of the indicated concentrations of miltirone or diazepam, after which the extent of specific radioligand binding was determined. Data are means (± S.E.M. of values from three independent experiments) and are expressed as a percentage of specific radioligand binding apparent in the absence of test drug. *$P < 0.01$ vs. respective control (100% binding).

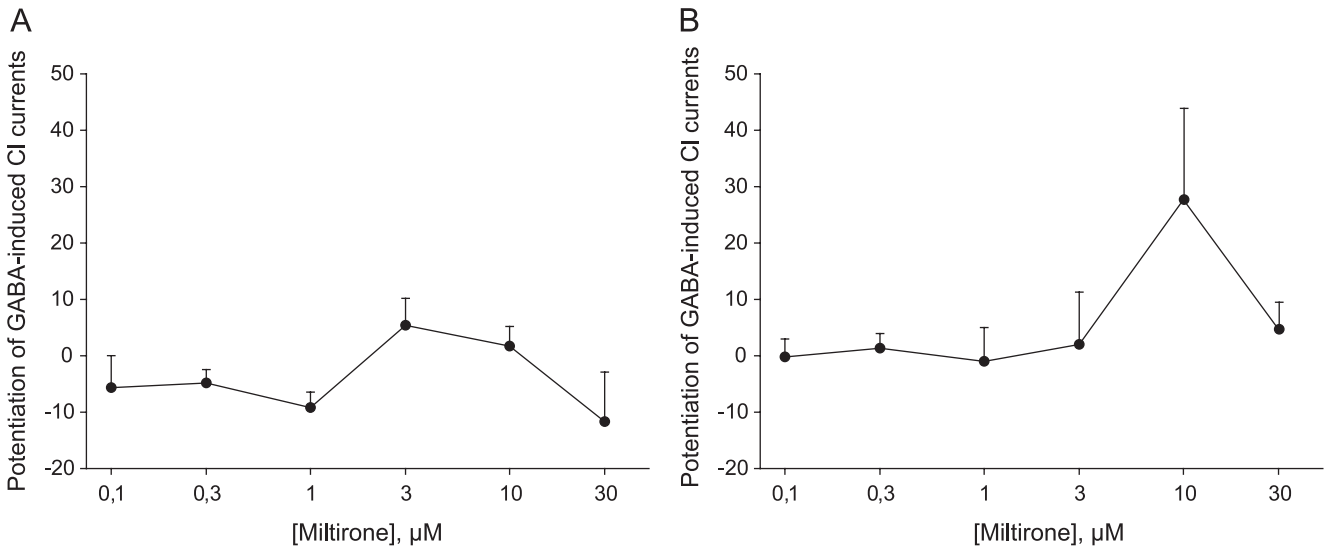

Fig. 2. Lack of effect of miltirone on GABA-induced Cl^- currents in *Xenopus* oocytes expressing recombinant human $GABA_A$ receptors. Oocytes expressing $\alpha_1\beta_2\gamma_2L$ (A) or $\alpha_2\beta_2\gamma_2L$ (B) receptors were exposed to GABA (EC_{5-10}) in the presence of the indicated concentrations of miltirone. Data are expressed as percentage potentiation of the control response to GABA and are means ± S.E.M. of values from three to five oocytes.

oocytes. Miltirone had no effect at any of the concentrations tested on Cl^- currents induced by GABA at a concentration that elicited 5–10% of the maximal response (EC_{5-10}, 5–10 μM) in oocytes expressing either receptor subtype (Fig. 2). However, miltirone (0.1–30 μM) did inhibit in a concentration-dependent manner the potentiation of $GABA_A$ receptor function by diazepam (1 μM); the IC_{50} values were 0.24 ± 0.22 and 0.618 ± 0.35 μM for $\alpha_1\beta_2\gamma_2L$ (Fig. 3A) and $\alpha_2\beta_2\gamma_2L$ (Fig. 3B) receptors, respectively.

3.3. Effects of miltirone on native $GABA_A$ receptors in cultured hippocampal neurons

We further assessed the effects of miltirone on $GABA_A$ receptor-mediated Cl^- currents in cultured rat hippocampal pyramidal cells with the whole-cell patch clamp technique. Miltirone (0.3–10 μM) did not significantly affect the amplitude of GABA-evoked Cl^- currents (Fig. 4A,B). Similar to its effect at recombinant $GABA_A$ receptors, however, miltirone (0.3–1 μM) inhibited in a concentra-

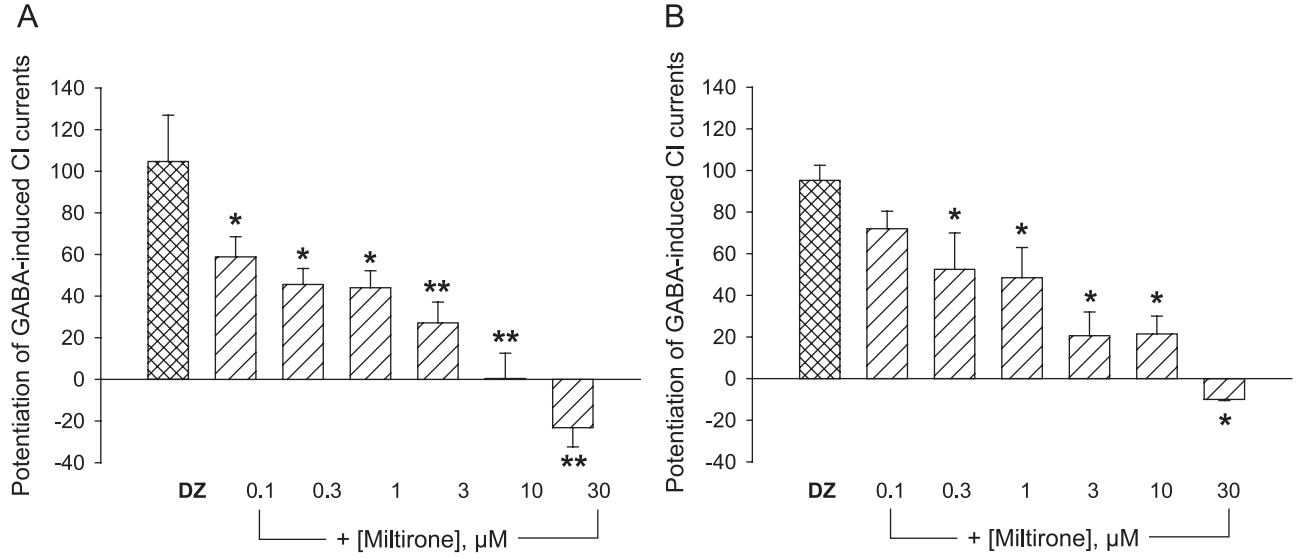

Fig. 3. Inhibition by miltirone of the diazepam-induced potentiation of GABA-evoked Cl^- currents in *Xenopus* oocytes expressing human recombinant $GABA_A$ receptors. Oocytes expressing $\alpha_1\beta_2\gamma_2L$ (A) or $\alpha_2\beta_2\gamma_2L$ (B) receptors were exposed to GABA (EC_{5-10}) and diazepam (1 μM) in the presence of the indicated concentrations of miltirone. Data are expressed as percentage potentiation of the control response obtained with GABA and are means ± S.E.M. of values from two to six oocytes. *$P < 0.05$, **$P < 0.01$ vs. value for diazepam without miltirone.

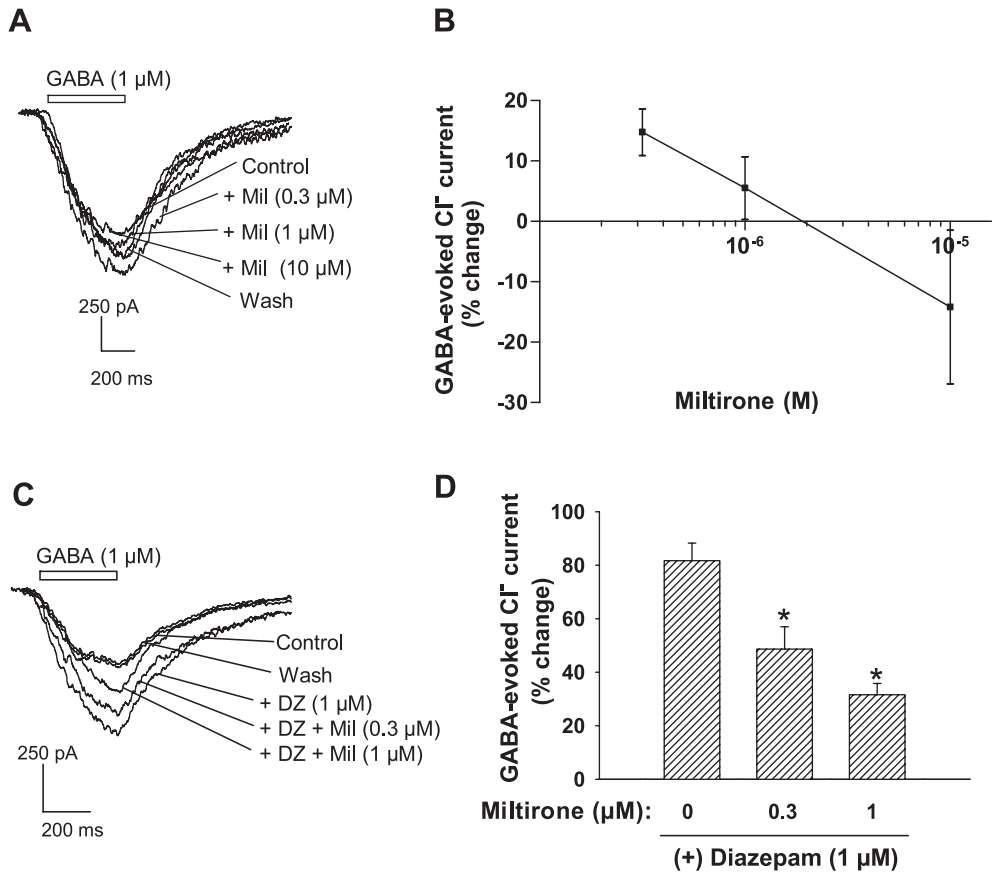

Fig. 4. Inhibition by miltirone of the diazepam-induced potentiation of GABA-evoked Cl^- currents in cultured hippocampal pyramidal cells. (A) Representative electrophysiological traces recorded from a single neuron showing the lack of effect of various concentrations of miltirone (Mil) on Cl^- currents evoked by 1 μM GABA. Control and wash represent the effects of GABA alone before and after its coapplication with miltirone, respectively (B) Quantitation of results similar to those shown in A. Data are expressed as percentage change in the GABA response and are means ± S.E.M. of values from three to five neurons. (C) Representative traces of Cl^- currents evoked by 1 μM GABA in the absence or presence of 1 μM diazepam (DZ) or 0.3 or 1 μM miltirone. (D) Quantitation of results similar to those shown in C. Data are expressed as percentage change in the GABA response and are means ± S.E.M. of values from three or four neurons. *$P<0.05$ vs. the effect of diazepam alone.

tion-dependent manner the potentiation of GABA-evoked Cl^- currents by 1 μM diazepam (Fig. 4C,D).

3.4. Effect of miltirone on the ethanol-withdrawal-induced increase in the abundance of the α_4 subunit mRNA

Consistent with previous observations (Sanna et al., in press), withdrawal of ethanol for 3 h from cultured hippocampal neurons that had been incubated with 100 mM ethanol for 6 days resulted in a marked increase in the abundance of the mRNA for the α_4 subunit of the $GABA_A$ receptor (Fig. 5). This effect of ethanol withdrawal was abolished by exposure of the neurons to diazepam (10 μM) at the time of ethanol discontinuation; diazepam per se did not affect the amount of this subunit mRNA in neurons not previously exposed to ethanol. Similar to the action of diazepam, miltirone (1 or 10 μM) significantly, but not completely, inhibited the increase in the abundance of the α_4 subunit mRNA induced by ethanol withdrawal (Fig. 5). Miltirone (10 μM) alone also had no effect on the amount of the α_4 subunit mRNA in neurons not exposed to ethanol. Furthermore, withdrawal of miltirone for 3 or 6 h after incubation of hippocampal neurons with the drug (10 μM) for 6 days had no effect on the abundance of the α_4 subunit mRNA (data not shown), in contrast to the effect of diazepam withdrawal under these conditions that increases the amount of this subunit mRNA (Follesa et al., 2002).

4. Discussion

We have shown that miltirone, a tanshinone isolated from the root of *S. miltiorrhiza* that binds to benzodiazepine receptors partially inhibited the increase in the abundance of the mRNA for the α_4 subunit of the $GABA_A$ receptor elicited by ethanol withdrawal in cultured hippocampal cells. Consistent with our previous results (Follesa et al., 2003; Sanna et al., in press), diazepam abolished this effect of ethanol withdrawal. The lower efficacy of miltirone compared with that of diazepam in this regard is consistent with the failure of miltirone to potentiate GABA-evoked Cl^- currents mediated by two subtypes

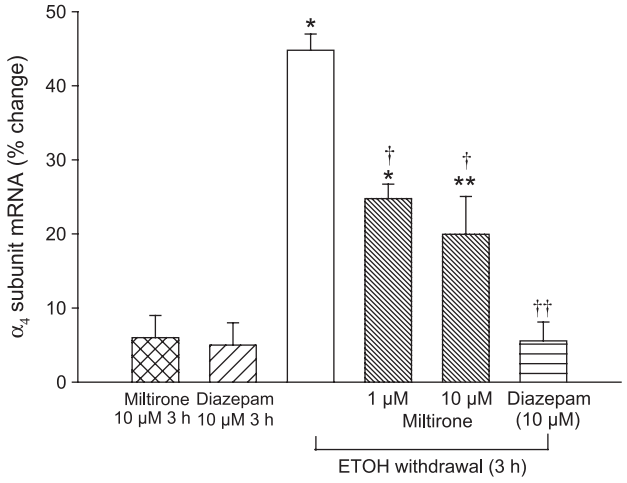

Fig. 5. Inhibition by miltirone or diazepam of the increase in the abundance of the mRNA for the α_4 subunit of the $GABA_A$ receptor induced by ethanol withdrawal in hippocampal neurons. Cells were incubated first for 6 days with 100 mM ethanol and then for 3 h without ethanol in the absence or presence of miltirone (1 or 10 μM) or diazepam (10 μM). The amount of the mRNA for the α_4 subunit of the $GABA_A$ receptor was then 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 value for cultures not exposed to ethanol. $^*P<0.001$ vs. control; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.001$ vs. ethanol withdrawal.

($\alpha_1\beta_2\gamma_2$L and $\alpha_2\beta_2\gamma_2$L) of recombinant human $GABA_A$ receptors expressed in *Xenopus* oocytes or by native $GABA_A$ receptors in cultured rat hippocampal pyramidal cells. Together, these observations support the idea that miltirone is a low-affinity partial agonist–antagonist of central benzodiazepine receptors. This conclusion is also suggested by the ability of miltirone to inhibit in a concentration-dependent manner the potentiation of GABA-evoked Cl^- currents by diazepam at recombinant and native $GABA_A$ receptors expressed in oocytes and hippocampal neurons, respectively. Moreover, the finding that miltirone is almost devoid of intrinsic activity at $GABA_A$ receptors under basal conditions while is able to modulate $GABA_A$ receptor gene expression during ethanol withdrawal, a condition associated to altered receptor gene expression and reduced receptor function, is consistent with the notion that a reduction of $GABA_A$ receptor-mediated transmission enhances the efficacy of benzodiazepine receptor ligand with low intrinsic activity.

Given that up-regulation of the α_4 subunit during withdrawal of ethanol, progesterone, or other positive allosteric modulators of $GABA_A$ receptors is associated with a lower threshold of excitability of neurons and a greater sensitivity also to anxiogenic, proconvulsant, and convulsant drugs (Smith et al., 1998), the ability of miltirone to partially inhibit this change in receptor subunit expression induced by ethanol discontinuation in cultured hippocampal neurons suggests that this drug might prove efficacious in reducing the symptoms of ethanol withdrawal in vivo. This conclusion is consistent with previous data showing that miltirone possesses pharmacological efficacy in experimental paradigms related to anxiety and neuronal excitability that are associated to a reduction of $GABA_A$ receptor function (Chang et al., 1991; Lee et al., 1991).

Sudden discontinuation of chronic treatment of rats or mice with various allosteric modulators of $GABA_A$ receptors (ethanol, progesterone, benzodiazepine receptor ligands) or the interruption of long-term exposure of neuronal cultures to these drugs results in an increase in the abundance of the mRNA for the α_4 subunit of the $GABA_A$ receptor (Concas et al., 1998; Follesa et al., 2000, 2002; Smith et al., 1998). In cultured hippocampal neurons, the effect of ethanol withdrawal on the amounts of both α_4 protein and mRNA was prevented by the substitution of ethanol with diazepam (Sanna et al., in press). Data obtained in rats and mice have suggested that drugs able to promote $GABA_A$ receptor-mediated neurotransmission either directly or indirectly are also able to reduce or abolish ethanol withdrawal symptoms in humans (Addolorato et al., 2002; Lejoyeux et al., 1998). Benzodiazepine receptor ligands with low intrinsic activity, such as miltirone, thus have therapeutic potential for the prevention of alcohol withdrawal syndrome. Accordingly, Carai et al. (2003) have shown that extracts of *S. miltiorrhiza* modulate ethanol intake and withdrawal in alcohol-preferring sP rats.

The partial agonist profile of miltirone at central benzodiazepine receptors in certain experimental conditions may confer some advantages in terms of reduced side effects in comparison with benzodiazepines. Consistent with this conclusion is our observation that miltirone withdrawal did not increase the abundance of the α_4 subunit mRNA in cultured hippocampal neurons. In contrast, withdrawal of imidazenil, another partial agonist of the central benzodiazepine receptor (Serra et al., 1994), increased the amount of the α_4 subunit mRNA in cultured cerebellar granule cells (Follesa et al., 2002). Finally, given that the efficacy of benzodiazepine receptor agonists with regard to modulation of GABA-evoked Cl^- currents is increased when both GABAergic transmission and the threshold of neuronal excitability are reduced, miltirone might also prove useful as a pharmacological agent to treat individuals who are dependent on benzodiazepines. The low intrinsic efficacy of miltirone under basal conditions and its likely greater efficacy during benzodiazepine withdrawal may confer on this compound the ability to restore the physiological pattern of $GABA_A$ receptor activity after its alteration by long-term administration and sudden discontinuation of benzodiazepines.

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