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Article *in* Neuropharmacology · October 2006

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The muscle relaxant thiocolchicoside is an antagonist of $GABA_A$ receptor function in the central nervous system

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Received 19 January 2006; received in revised form 14 April 2006; accepted 17 May 2006

Abstract

Thiocolchicoside (TCC) is used clinically for its muscle relaxant, anti-inflammatory, and analgesic properties, and it has been shown to interact with γ-aminobutyric acid (GABA) type A receptors ($GABA_A$Rs) and strychnine-sensitive glycine receptors in the rat central nervous system. In contrast to a proposed agonistic action at these two types of inhibitory receptors, pharmacological evidence has shown that, under certain conditions, TCC manifests convulsant activity in animals and humans. We now show that the phasic and tonic $GABA_A$R-mediated currents recorded from Purkinje cells and granule neurons, respectively, in parasagittal cerebellar slices from adult male rats were inhibited by TCC in a concentration-dependent manner. The median inhibitory concentrations of TCC for these effects were ∼0.15 and ∼0.9 μM, respectively. TCC did not potentiate $GABA_B$R-mediated currents in hippocampal slices, suggesting that its muscle relaxant action is not mediated by $GABA_B$Rs. Intraperitoneal injection of TCC in rats either alone or in combination with negative modulators of GABAergic transmission revealed convulsant and proconvulsant actions of this drug. Our data, consistent with clinical observations of the epileptogenic effect of this compound, suggest that TCC is a potent competitive antagonist of $GABA_A$R function.

Keywords: GABA receptor; Epilepsy; Competitive antagonist; Cerebellum; Patch clamp; Tonic current

1. Introduction

Thiocolchicoside (TCC) (Fig. 1A) is a semisynthetic sulfur derivative of colchicoside, a naturally occurring glucoside present in the plant *Gloriosa superba*. TCC has been used clinically for more than 35 years as a muscle-relaxant, anti-inflammatory, and analgesic drug (Janbroers, 1987), but its molecular targets and mechanisms of action are still under investigation. This compound has been shown to inhibit the binding of [^{3}H]GABA (γ-aminobutyric acid) or [^{3}H]strychnine to rat cerebrocortical or spinal cord membranes, respectively, in vitro as well as to corresponding autoradiographic sections in vivo (Balduini et al., 1999, 2001; Cimino et al., 1996). [^{3}H]TCC was also displaced in a concentration-dependent manner by GABA or by several agonists or antagonists of the type A receptor for GABA ($GABA_A$R). Moreover, TCC was shown to interact preferentially with a subpopulation of $GABA_A$Rs with low-affinity binding sites for GABA (Balduini et al., 2001).

Although its precise mechanisms of action remain unknown, TCC has been thought to act as a $GABA_A$R agonist

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doi:10.1016/j.neuropharm.2006.05.023

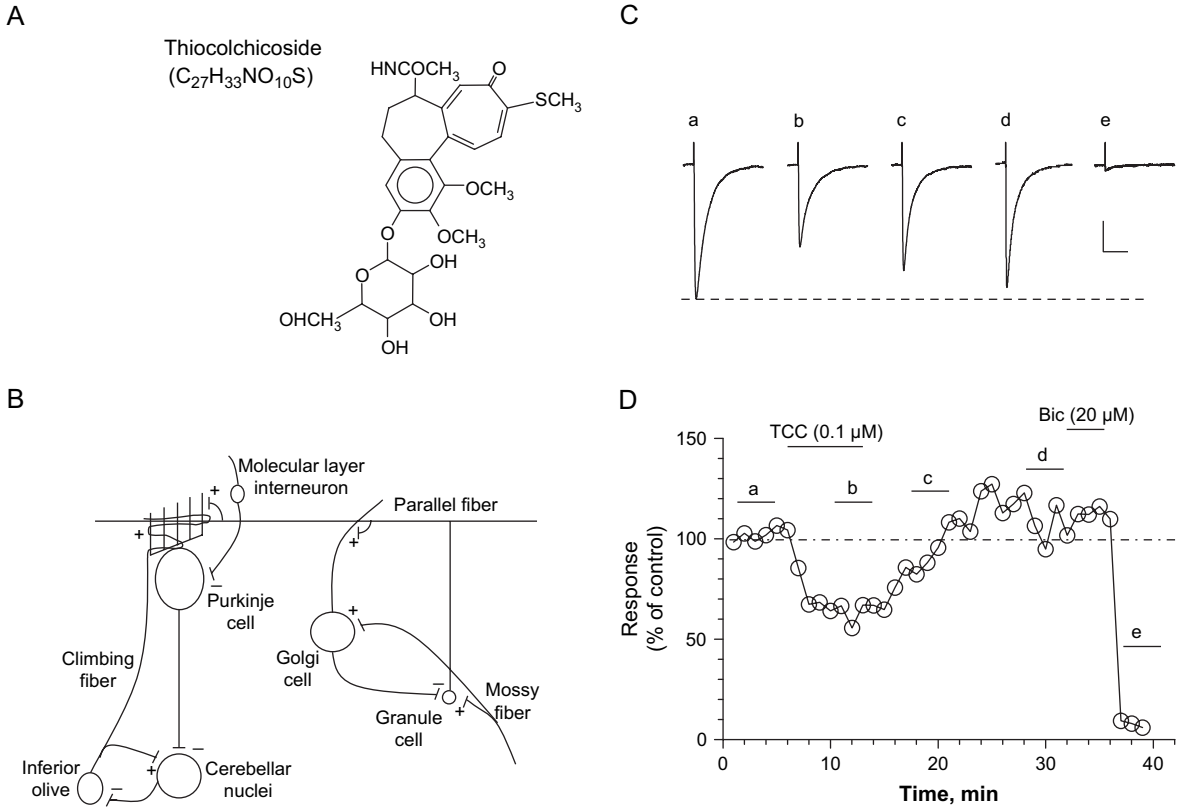

Fig. 1. Reversible inhibition by TCC of $GABA_AR$-mediated eIPSCs in cerebellar Purkinje neurons. (A) Molecular structure of thiocolchicoside (TCC). (B) Simplified representation of the cerebellar circuits relevant to the present study. Mossy fibers, which originate in the spinal cord and brain stem, provide excitatory inputs to granule and Golgi cells. Granule cells also receive inhibitory input from Golgi cells and provide excitatory inputs to Purkinje cells and molecular-layer interneurons via parallel fibers. Molecular-layer interneurons provide inhibitory input to Purkinje cells. (C) Averaged traces of three to five $GABA_AR$-mediated currents recorded from a single cerebellar Purkinje neuron. The eIPSCs were elicited by stimulation of molecular-layer interneurons near the recorded Purkinje cell in the presence of kynurenic acid (3 mM). Currents were recorded under control conditions (a), in the presence of 0.1 μM TCC (b), after washout of TCC for 5 to 10 min (c, d), and in the presence of 20 μM bicuculline (e). Scale bars: current amplitude, 200 pA; time, 100 ms. (D) Time course of the effects of TCC and bicuculline (Bic) on eIPSC amplitude in the same cell as that studied in (C). The times during which the traces (a) through (e) in (C) were recorded are indicated. Data are expressed as a percentage of the control response.

that induces depression of the central nervous system and, in turn, myorelaxation (Biziere et al., 1981). However, under specific experimental or clinical conditions, TCC has been shown to induce epileptic seizures (De Riu et al., 2001; Sechi et al., 2003), challenging the notion that this drug acts as an agonist at $GABA_ARs$ and raising the possibility that it may directly or indirectly inhibit GABAergic transmission. Moreover, TCC possesses a molecular structure similar to that of colchicine, a plant alkaloid that binds to tubulin and induces the depolymerization of microtubules (Osborn and Weber, 1976), disrupts axonal transport (Karlsson and Sjostrand, 1969), and inhibits mitosis (Wilson and Friedkin, 1966). Colchicine induces epileptic seizures in rodents (Dasheiff and Ramirez, 1985), and its intracranial administration triggers generalized convulsions and death (Wisniewski and Terry, 1967). In addition to its well-characterized effects on microtubules, colchicine acts directly as a competitive antagonist of $GABA_AR$ function (Weiner et al., 1998), possibly explaining, at least in part, its epileptogenic action.

In view of these contrasting observations with TCC, we have now investigated the effects of this drug on the function of native $GABA_ARs$ in cerebellar slices with the patch-clamp technique. Purkinje neurons and cerebellar granule cells of adult rats manifest a relatively uniform expression of $GABA_AR$ isoforms. In particular, Purkinje neurons express mostly $GABA_ARs$ that contain the α1, β2 or β3, and γ2 subunits; these receptors exhibit a relatively low affinity for GABA and are localized in the synaptic cleft (Pirker et al., 2000; Wisden et al., 1996). In contrast, granule cells, in their extrasynaptic regions, express $GABA_ARs$ that mostly comprise the α6, β2 or β3, and δ subunits, exhibit a higher affinity for GABA, and mediate the tonic component of inhibitory transmission. In addition to these extrasynaptic receptors, granule cells express synaptic $GABA_ARs$ that contain α1 or α6, β2 or β3, and γ2 subunits; these low-affinity receptors bind GABA that is released from Golgi cells and mediate fast-rising spontaneous inhibitory postsynaptic currents (sIPSCs) (Brickley et al., 1996, 2001; Hamann et al., 2002; Rossi and Hamann, 1998; Rossi et al., 2003; Tia et al., 1996; Wall and Usowicz, 1997). We now show that TCC is a potent competitive antagonist of $GABA_AR$ function. This compound shows a greater potency at the synaptic $GABA_ARs$

of Purkinje cells than at the extrasynaptic $GABA_A$Rs of granule cells. Consistent with our electrophysiological observations, in vivo experiments revealed that TCC acts as a convulsant and proconvulsant drug. Our results are thus in accord with the previous demonstration of focal and secondarily generalized convulsive epileptic activity of TCC in rats and humans (De Riu et al., 2001; Sechi et al., 2003).

2. Methods

2.1. Animals

Male Sprague–Dawley CD rats were obtained from Charles River (Como, Italy). After arrival at the animal facility, rats were allowed to acclimatize to the new housing conditions for at least 1 week. They were housed six per cage under an artificial 12-h-light, 12-h-dark cycle (lights on from 08:00 to 20:00 h) and at a constant temperature of 22° ± 2 °C and relative humidity of 65%. They had free access to water and standard laboratory food at all times. Animal care and handling throughout the experimental procedures 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.

2.2. Preparation of brain slices and electrophysiological recording

Rats (28 to 40 days old) were anesthetized in a chamber saturated with chloroform and then decapitated. The brain was removed rapidly and placed in an ice-cold solution containing 220 mM sucrose, 2 mM KCl, 1.3 mM NaH_2PO_4, 12 mM $MgSO_4$, 0.2 mM $CaCl_2$, 10 mM glucose, and 2.6 mM $NaHCO_3$ (pH 7.3, equilibrated with 95% O_2 and 5% CO_2). Coronal hippocampal slices (thickness, 300 μm) and parasagittal cerebellar slices (thickness, 200 to 250 μm) were prepared with a Vibratome 1000 Plus instrument (Vibratome, St. Louis, MO) and then incubated first for 40 min at 34 °C and then for 30 min at room temperature in artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose, and 26 mM $NaHCO_3$ (pH 7.3, equilibrated with 95% O_2 and 5% CO_2).

Slices were transferred to a chamber perfused with ACSF at a rate of ~2 ml/min and at room temperature. Whole-cell patch-clamp electrophysiological recordings were performed with an Axopatch 200-B amplifier (Axon Instruments, Union City, CA) and an infrared-differential interference contrast microscope. Patch microelectrodes (borosilicate capillaries with a filament and an outer diameter of 1.5 μm; Sutter Instruments, Novato, CA) were prepared with a two-step vertical puller (Sutter Instruments) and had a resistance of 4 to 6 MΩ. Evoked inhibitory postsynaptic currents (eIPSCs) as well as tonic GABAergic currents were recorded at a holding potential of −65 mV with an internal solution containing 140 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM HEPES-CsOH (pH 7.3), 2 mM ATP (disodium salt), and 5 mM QX-314 (lidocaine *N*-ethyl bromide). $GABA_B$R-mediated currents were recorded at a holding potential of −50 mV with an internal solution containing 4 mM KCl, 0.1 mM EGTA, 10 mM HEPES-KOH (pH 7.3), 140 mM potassium gluconate, and 2 mM ATP (magnesium salt). All GABAergic currents were recorded in the presence of kynurenic acid (3 mM) in the external solution. Access resistance varied between 20 and 40 MΩ; if it changed by >20% during an experiment, the recording was discarded. Currents through the patch-clamp amplifier were filtered at 2 kHz and digitized at 5.5 kHz with commercial software (pClamp 8.2, Axon Instruments). In Purkinje cells, eIPSCs were elicited with a concentric bipolar stimulating electrode placed 100 to 200 μm from the patched neuron. Pairs of stimuli were delivered at 300-ms intervals every 20 s (frequency of 0.05 Hz). For analysis of tonic GABAergic currents, we used parameters described previously (Carta et al., 2004). In brief, we selected epochs of 3 s every 30 s of recording and visually excluded sIPSCs from the analysis. For the analysis of sIPSCs, we visually selected all events, which are readily distinguishable from the background noise on the basis of their characteristic fast rise times and slower decay times. Evoked $GABA_B$R-mediated currents were elicited by placing the stimulating electrode 100 to 200 μm from the patched CA1 hippocampal pyramidal neuron; a single stimulus was delivered every 30 s. Unless indicated otherwise, each slice was exposed only once to a single TCC concentration. The effect of TCC was quantified with respect to the average of control responses obtained before drug application.

2.3. Primary culture of hippocampal neurons and electrophysiological recording

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, San Diego, CA) 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, and the resulting fragments were 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 in 24 well dishes (6×10^5 cells per well) containing 12-mm-diameter round glass coverslips coated with poly-L-lysine. Cells were cultured in a humidified incubator containing 5% CO_2 at 37 °C. Twenty-four hours after plating, fetal bovine serum was replaced with B-27 supplement (Invitrogen), and glutamate was removed from the medium after 3 days of culture.

Immediately before recording, coverslips were transferred to a perfusion chamber (Warner Instruments, Hampden, CT), and neurons were visualized 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. Preparation of the recording pipettes and the internal solution were as described for slice recording. 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. Drugs were applied with a fast-exchange flow-tube perfusion system driven by a motor (Warner Instruments). GABA (0.5, 1, 10, 30, 100 μM) was applied at intervals of 30 s. All experiments were performed at room temperature (23 to 25 °C). Data were analyzed by pClampfit 8.01 software (Axon Instruments). Modulation of GABA-evoked Cl^- currents by TCC was expressed as percentage change, $[(I'/I) - 1] \times 100\%$, where I is the average of control responses obtained before drug application, and I' is the average of the GABA-induced responses obtained from the same cell in the presence of drug.

2.4. Evaluation of proconvulsant and convulsant activity

Rats with body masses of 150 to 180 g were divided into groups of three to nine for each pharmacological treatment. In one set of experiments, animals were injected intraperitoneally (i.p.) with various doses of TCC from 10 to 30 mg per kilogram of body mass. In a second set of experiments, TCC (10 or 15 mg/kg, i.p.) was administered 30 min before the injection of pentylenetetrazol (PTZ) at doses of 35, 45, or 55 mg/kg (i.p.).

In a third set of experiments, TCC (15 mg/kg, i.p.) was administered 30 min prior to the injection of the proconvulsant β-carboline FG 7142 (*N*-methyl-β-carboline-3-carboxamide) at 25 mg/kg (i.p.). All treated animals were monitored for at least 60 min after drug administration for determination of the seizure onset time (time between drug administration and the appearance of convulsions), the number of animals that manifested convulsions, and the number of deaths. The pattern of seizure activity, which usually consisted of muscular contractions (twitches), wild running, and tonic and clonic convulsions, was also monitored.

2.5. Drugs

A stock solution of 10 mM TCC (kindly provided by Inverni Della Beffa, Limito, Milano, Italy) was prepared in distilled water. Stock solutions of bicuculline methiodide (Sigma) and SCH 50911 (Tocris, Bristol, UK) were prepared in dimethyl sulfoxide (maximal final concentration of 0.03%) and

distilled water, respectively. Kynurenic acid (Sigma) was dissolved directly in ACSF at 3 mM. FG 7142 (kindly provided by Schering A.G., Berlin, Germany) was dissolved in saline with one drop of Tween 80 per 3 ml of solution. PTZ (Sigma) was dissolved in physiological saline.

2.6. Statistical analysis

Data are presented as means of absolute value or, as mentioned, of percentage of change ± SEM. Statistical comparisons of pooled data of absolute value were performed by Student's *t*-test with the use of Prism software (Graph-Pad, San Diego, CA). A P value of <0.05 was considered statistically significant. Non-linear regression analysis of TCC concentration-response relations was also performed with Prism software. Schild plot was constructed from measuring dose-ratios obtained from the displacements in the GABA concentration-response curves in the presence of increasing concentrations of TCC.

3. Results

3.1. Effect of TCC on GABAergic currents in cerebellar Purkinje neurons

We first examined the effects of TCC on the function of $GABA_A$Rs in Purkinje and granule neurons present in thin cerebellar slices. We recorded eIPSCs in Purkinje cells, in which most synaptic $GABA_A$Rs comprise α1, β2 or β3, and γ2 subunits (Pirker et al., 2000; Wisden et al., 1996). The eIPSCs were elicited with a bipolar concentric stimulating electrode placed in the molecular layer near the target neuron (Fig. 1B). Kynurenic acid (3 mM) was added to the external solution in order to block the various types of ionotropic glutamate receptors, thereby isolating $GABA_A$R-mediated currents. The eIPSCs were characterized by a fast rise time (7.31 ± 2.1 ms, $n = 29$) and by a slower decay time (69.8 ± 4.8 ms, $n = 29$), with an amplitude in the range of 300 to 1000 pA and with an average of 370.3 ± 35.5 pA ($n = 29$). Bath application of TCC (0.1 μM) induced a reversible inhibition of the GABAergic synaptic currents (Fig. 1C, D). The $GABA_A$R antagonist bicuculline (20 μM) completely blocked all eIPSCs, confirming that they were mediated by $GABA_A$Rs (Fig. 1C, D). The inhibitory effect of TCC was fully reversible at concentrations of ≤1 μM, but was only partially reversible, even after several minutes of washout, at concentrations of >1 μM (data not shown).

TCC (0.001 to 100 μM) reduced the amplitude of eIPSCs in a concentration-dependent manner (Fig. 2), with this effect being significant at 0.1 μM (−50 ± 9%) and maximal at 10 μM (−96 ± 2%). Non-linear regression analysis yielded an apparent median inhibitory concentration (IC_{50}) of 145 nM [95% confidence interval (CI), 51 to 408 nM], with a Hill slope of 0.44 (95% CI, 0.233 to 0.653).

We also examined whether TCC affected the kinetic parameters of eIPSCs. Whereas TCC at concentrations of 0.001, 0.1, or 1 μM had no significant effect on the rise time of eIPSCs, it slightly but significantly reduced the decay time at 1 μM but not at 0.1 or 0.001 μM (Fig. 3A, B). We were not able to analyze the effects of TCC at concentrations of >1 μM because of the complete inhibition of $GABA_A$R-mediated currents apparent at such concentrations.

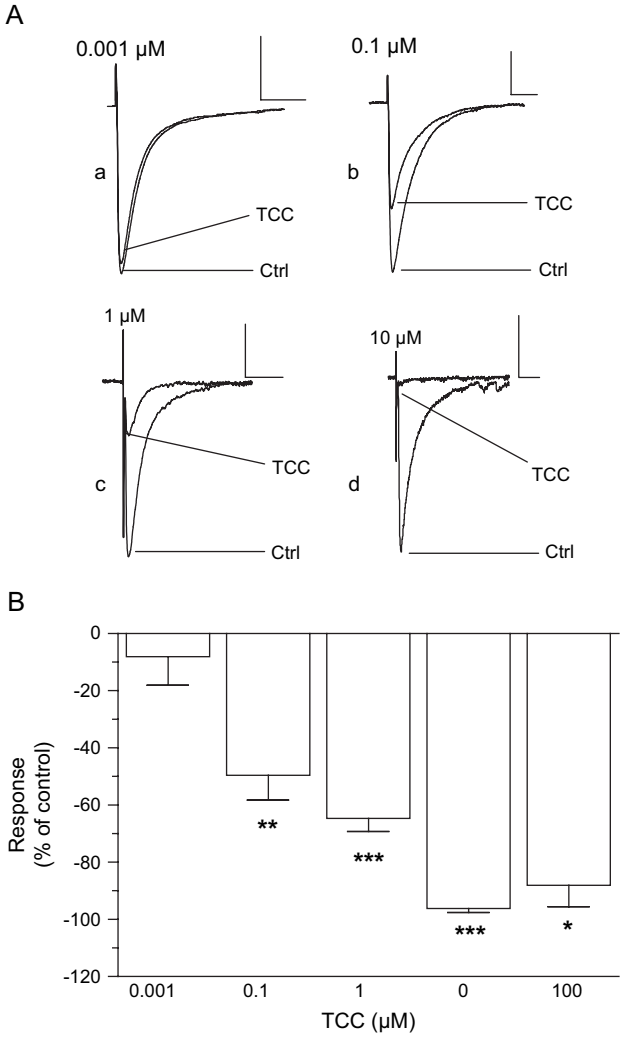

Fig. 2. Concentration dependence of the inhibitory effect of TCC on $GABA_A$R-mediated eIPSCs in cerebellar Purkinje cells. (A) Averages of four traces showing the effect of various concentrations of TCC on eIPSCs recorded from different Purkinje cells. Bicuculline (20 μM) was applied to all cells to confirm that the recorded currents were mediated by $GABA_A$Rs (not shown, for clarity). Ctrl, Control traces. Scale bars: current amplitude, 400 pA (a) and 200 pA (b–d); time, 50 ms. (B) Summary of the effects of TCC at 0.001 μM ($n = 11$), 0.1 μM ($n = 9$), 1 μM ($n = 9$), 10 μM ($n = 5$), and 100 μM ($n = 4$) on eIPSC amplitude. Data are expressed as percentage change relative to the control response and are means ± SEM. $*P < 0.005$, $**P < 0.001$, $***P < 0.0005$ versus the hypothetical mean of zero (one-sample t test).

We next evaluated whether the inhibitory effect of TCC on eIPSCs might be exerted at the presynaptic level through inhibition of GABA release. For these experiments, we applied a paired-pulse protocol to study the function of presynaptic terminals. When synaptic responses are elicited by two stimuli delivered with a brief interpulse interval and at the same intensity, the amplitude of the second response depends on the probability of neurotransmitter release. We placed the stimulating electrode near (within 50 to 100 μm of) the recorded Purkinje cell in order to stimulate the axons of the surrounding interneurons. Under these conditions, the paired-pulse ratio (ratio of the amplitude of the second response to that of the

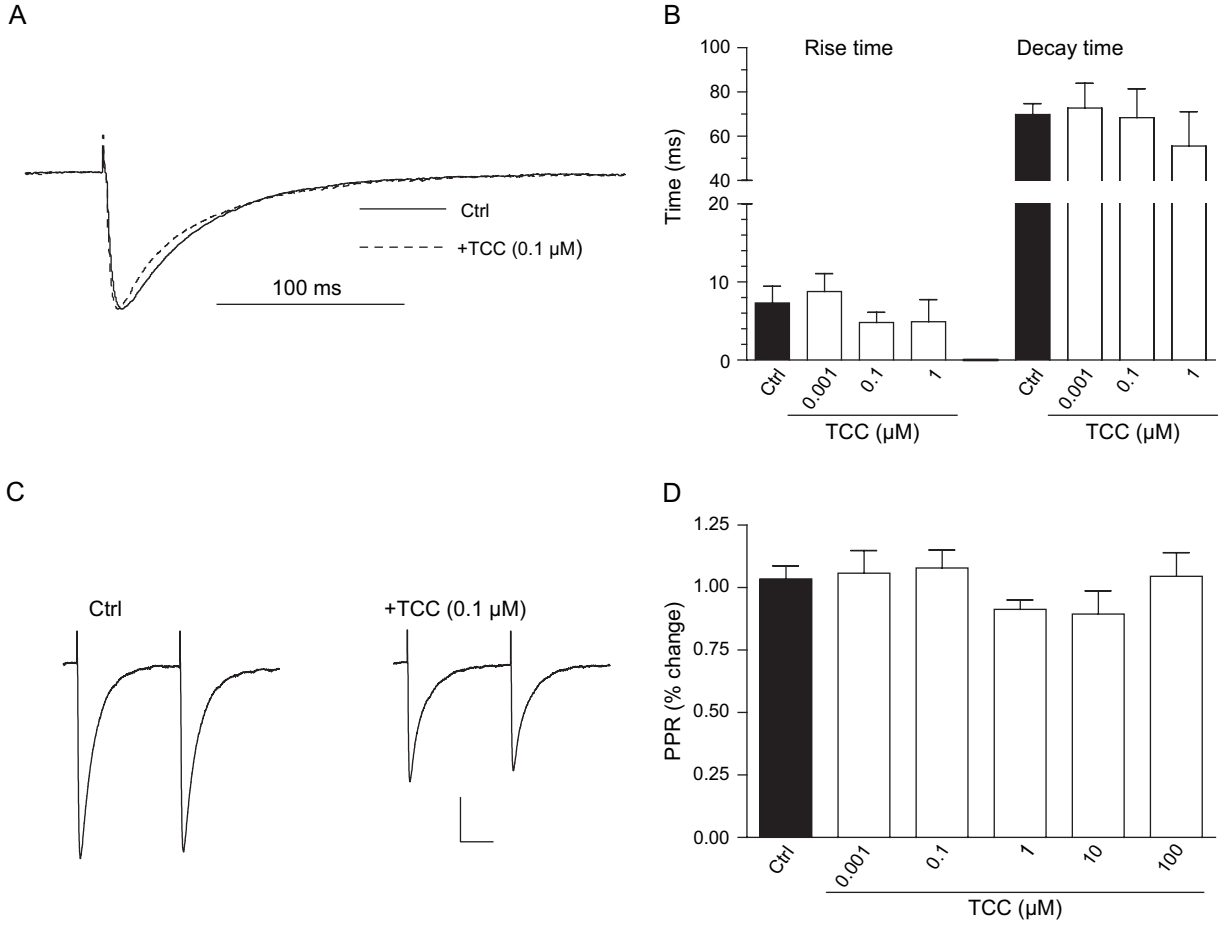

Fig. 3. Effects of TCC on the rise and decay time constants of eIPSCs and on the paired-pulse ratio in cerebellar Purkinje neurons. (A) Superimposed and scaled traces of eIPSCs (average of three to five responses) recorded from the same Purkinje cell under control conditions and in the presence of TCC (0.1 μM). (B) Summary of the effects of TCC at 0.001 μM ($n = 9$), 0.1 μM ($n = 9$), and 1 μM ($n = 8$) on the rise and decay times of eIPSCs. Data are absolute values and are expressed as means ± SEM. (C) Averages traces for four pairs of eIPSCs (interpulse interval, 300 ms) recorded from Purkinje cells under control conditions and in the presence of TCC (0.1 μM). Scale bars: current amplitude, 200 pA; time, 100 ms. (D) Summary of the effects of TCC at 0.001 μM ($n = 11$), 0.1 μM ($n = 9$), 1 μM ($n = 9$), 10 μM ($n = 5$), and 100 μM ($n = 4$) on the paired-pulse ratio (PPR) for eIPSCs. Data are absolute values and are expressed as means ± SEM.

first response) was 1.035 ± 0.05 ($n = 29$, data not shown). TCC at concentrations of 0.001 to 100 μM had no significant effect on the paired-pulse ratio (Fig. 3C, D), suggesting that TCC does not act at the presynaptic level and that its inhibition of eIPSCs is mediated postsynaptically.

3.2. Effect of TCC on GABAergic currents in cerebellar granule cells

The possible effect of TCC on tonic inhibitory GABAergic currents in cerebellar granule cells was next investigated. These neurons express $GABA_A$Rs composed of α6, β2 or β3, and δ subunits in their extrasynaptic regions (Brickley et al., 1996, 2001; Hamann et al., 2002; Rossi and Hamann, 1998; Rossi et al., 2003; Wall and Usowicz, 1997). These receptors possess a high affinity for GABA that is released from Golgi cells and spills over from the synaptic cleft, and they mediate a non-desensitizing type of inhibitory current. In addition, the GABA released from Golgi cell axons activates synaptic $GABA_A$Rs that are composed mostly of α1 or α6, β2 or β3, and γ2 subunits and which generate sIPSCs (Brickley et al., 1996, 2001; Hamann et al., 2002; Rossi and Hamann, 1998; Rossi et al., 2003; Tia et al., 1996; Wall and Usowicz, 1997).

Consistent with previous observations (Brickley et al., 1996, 2001; Carta et al., 2004; Wall and Usowicz, 1997), we found that granule cells exhibited a tonic GABAergic current that was abolished by a saturating concentration of bicuculline (20 μM). In the presence of kynurenic acid (3 mM), the average size of the outward current induced by the application of bicuculline (20 μM) was 28.95 ± 6.1 pA ($n = 11$), whereas the percentage reduction in the noise variance was 62.97 ± 5.29 ($n = 11$) (Fig. 4A, B). The application of TCC (0.1 μM) had no significant effect on either the holding current or the current noise variance (Fig. 4C). After a washout period of ≥10 min, bath application of TCC (10 μM) markedly reduced both the holding current and the noise variance. This effect of TCC was not completely reversible. Finally, application of bicuculline (20 μM) blocked all residual current. The effects of TCC on both the holding current (Fig. 4D) and the

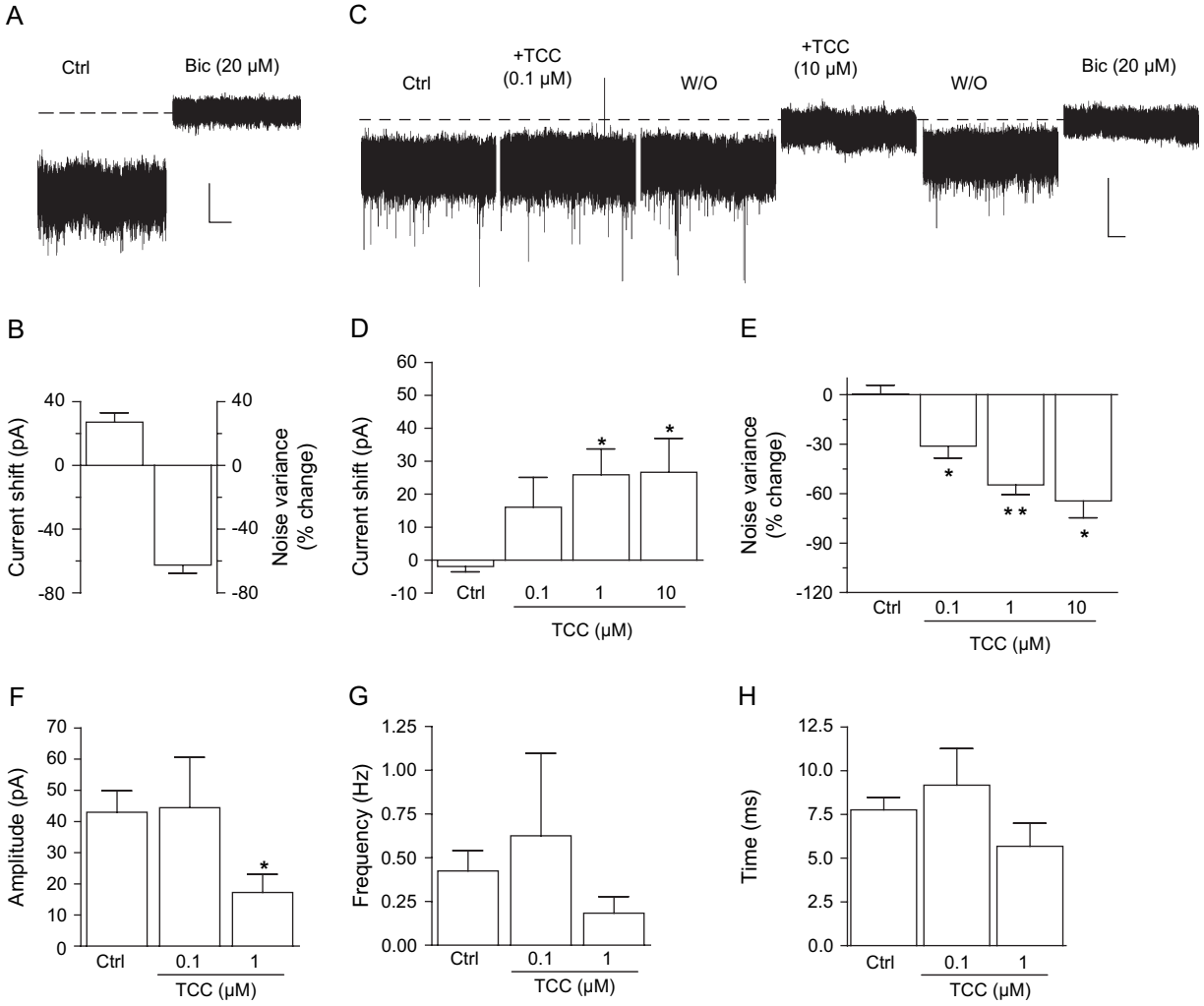

Fig. 4. Inhibition by TCC of tonic GABAergic currents in cerebellar granule neurons. (A) Representative traces of tonic GABAergic currents recorded from a cerebellar granule neuron under control conditions and during the application of bicuculline (20 μM). Scale bars: current amplitude, 20 pA; time, 10 ms. (B) Summary of the effects of bicuculline (20 μM) on the holding current (current shift) and current noise variance. Data are means ± SEM ($n = 11$). (C) Representative traces of the effect of TCC on tonic GABAergic currents. TCC was applied at concentrations of 0.1 and 10 μM with a washout (W/O) period of about 10 min between the two applications. Bicuculline (20 μM) was applied after subsequent washout of TCC at the higher concentration. Scale bars: current amplitude, 20 pA; time, 10 ms. (D, E) Summary of the effects of TCC at 0.1 μM ($n = 5$), 1 μM ($n = 7$), 10 μM ($n = 5$), and 100 μM ($n = 6$) on the tonic holding current and noise variance, respectively. Data are means ± SEM. $^*P < 0.05$, $^{**}P < 0.005$, versus the hypothetical mean of zero (one-sample t test). (F, G, H) Summary of the effects of TCC at 0.1 μM ($n = 4$), and 1 μM ($n = 6$) on the amplitude, frequency and decay time of the sIPSCs, respectively. Data are absolute values and are expressed as means ± SEM. $^*P < 0.05$, one-sample t test vs control value.

noise variance (Fig. 4E) were concentration dependent. Non-linear regression analysis revealed that the IC_{50} for the effect of TCC on the holding current was 0.89 μM (95% CI, 10.9 nM to 5.89 μM), with a Hill slope of 1.42 (95% CI, −13.1 to 15.94), whereas that for the effect on noise variance was 0.99 μM (95% CI, 0.39 to 2.5 μM), with a Hill slope of 1.32 (95% CI of −1.2 to 3.8). All tonic GABAergic currents were blocked by bicuculline (20 μM), confirming that they were mediated by $GABA_A$Rs.

Embedded in the tonic current, recordings from most (17 out of 23) granule cells were detectable sIPSCs with an average frequency of 0.42 ± 0.11 Hz, amplitude of 42.9 ± 6.9 pA, and a decay time of 6.24 ± 0.71 ms. Application of TCC at 0.1 μM ($n = 4$), 1 μM ($n = 6$), 10 μM ($n = 5$), and 100 μM ($n = 2$) induced a concentration-dependent reduction in the amplitude of sIPSCs. TCC, at 0.1 μM and 1 μM, changed the amplitude of sIPSCs to 44.4 ± 16.2 and 17.2 ± 5.8 pA, respectively (Fig. 4F). The inhibitory effect of TCC was significant ($p < 0.05$) at 1 μM and the blockade of sIPSCs was complete at the concentration of 10 μM. The apparent frequency of sIPSCs was also reduced by TCC at concentrations of ≥1 μM, but this effect could be most likely attributable to a decrease in sIPSC amplitude (Fig. 4G), and not to a decrease in frequency per se. Finally, TCC at all the concentrations tested failed to affect the decay time values of the sIPSCs (Fig. 4H).

3.3. Effect of TCC on GABAergic currents in cultured hippocampal neurons

To determine whether the inhibition of $GABA_A$R function by TCC was competitive or allosteric in nature, we performed electrophysiological recordings from cultured hippocampal

neurons. We tested the effect of three different concentrations of TCC (0.1, 0.5 and 1 μM) on the Cl^- currents evoked by increasing concentrations of GABA (0.3 to 100 μM). The inhibitory effect of TCC on these currents decreased as the GABA concentration increased (Fig. 5). TCC (0.1, 0.5 and 1 μM) significantly shifted the GABA concentration-response curves increasing the GABA EC_{50} from 6.1 ± 1.1 μM to 7.9 ± 1.8 μM, 18.66 ± 1.18 μM, and 40.35 ± 1.12 μM respectively. The Hill coefficient, from a control value of 0.99 ± 0.2647 was not significantly changed in the presence of TCC (0.1 μM, 0.9007 ± 0.2973; 0.5 μM, 0.9118 ± 0.3556; 1 μM, 1.003 ± 0.2558). The Schild plot of log(dose ratio − 1) against log TCC concentration resulted in a fitted line with a slope value of 1.21 ± 0.23 (Fig. 5B).

3.4. Effect of TCC on $GABA_BR$-mediated currents in hippocampal CA1 pyramidal neurons

Given that baclofen, a selective $GABA_BR$ agonist, is used clinically as a muscle relaxant (Bowery et al., 2002; Enna, 1997), we examined whether the observed myorelaxant effect of TCC might be mediated by interaction with $GABA_BRs$. The $GABA_BR$ is a metabotropic receptor that, on stimulation with GABA, activates a K^+ channel in the postsynaptic neuron and thereby induces hyperpolarization (Newberry and Nicoll, 1984). We recorded evoked $GABA_BR$-dependent K^+ currents from visualized CA1 pyramidal neurons by electrically stimulating interneurons in the stratum radiatum (Fig. 6A). We studied hippocampal CA1 pyramidal cells because of the lack of a prominent $GABA_BR$-mediated current in Purkinje neurons. For these experiments, we used a potassium gluconate-based solution in the recording electrode and clamped the cells at −50 mV in the presence of kynurenic acid (3 mM) and bicuculline (20 μM). Most $GABA_BR$-mediated currents had an amplitude of 22.8 ± 1.5 pA ($n = 21$) and were characterized by slow rise and decay times with an area of 5719 ± 577 pAms ($n = 21$) (Fig. 6B), consistent with the kinetics of metabotropic receptor-mediated signal transduction (Solis and Nicoll, 1992). TCC 0.1, 1, and 10 μM changed the area of the $GABA_BR$-mediated current to 5808 ± 450, 4753 ± 750, and 4104 ± 823 pAms, respectively. Statistical analysis failed to reveal any significant difference among groups. All the currents were blocked by the $GABA_BR$-selective antagonist SCH 50911 at 20 μM. Similar results were obtained by measuring the peak amplitude of $GABA_BR$-mediated currents (data not shown).

3.5. Proconvulsant and convulsant actions of TCC in healthy rats

Previous in vivo studies showed that, under certain conditions, TCC induced focal and secondarily generalized convulsive epileptic seizures in both animal models and humans (De Riu et al., 2001; Sechi et al., 2003). Our in vitro data showing that TCC is a competitive inhibitor of $GABA_AR$ function suggested that this drug might have a convulsant or proconvulsant profile. To evaluate this hypothesis, we tested the effects of various doses of TCC administered to rats either alone or together with the β-carboline FG 7142 (a proconvulsant drug) or PTZ (a convulsant drug).

The i.p. administration of TCC (10 to 30 mg/kg) revealed that the drug induced tonic-clonic seizures at doses of ≥20 mg/kg (Table 1). At 20 mg/kg, TCC induced convulsions in two out of five (40%) animals with a delay of 29 ± 2 min. In general, the seizures were characterized by a clonic component, affecting both the anterior and posterior limbs, and by a more marked tonic component, with extension and rigidity of limbs. The latency of seizures decreased as the dose of TCC increased. At a dose of 30 mg/kg, TCC induced seizures and death in all animals tested.

PTZ, a non-competitive antagonist of the $GABA_AR$ that acts at the picrotoxin site in the associated Cl^- channel, exerts convulsant activity in rats when injected i.p. at doses of

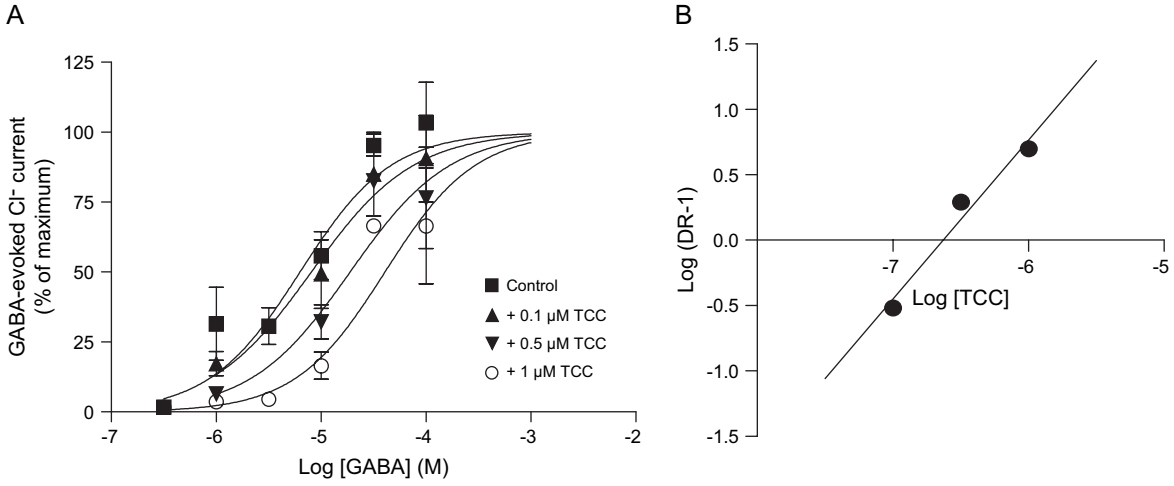

Fig. 5. Competitive inhibition by TCC of GABA-evoked Cl^- currents in cultured rat hippocampal neurons. (A) Summary of the effect of TCC 0.1 μM ($n = 3$), 0.5 μM ($n = 5$) and 1 μM ($n = 7$) on the GABA concentration- response curve. Each point represents the mean ± SEM from 3 to 6 cells tested at each concentration. (B) Schild plot obtained for the interaction of TCC (0.1, 0.5 and 1 μM) on the GABA concentration-response curve, with a slope of 1.21 ± 0.23, compatible with a competitive antagonism.

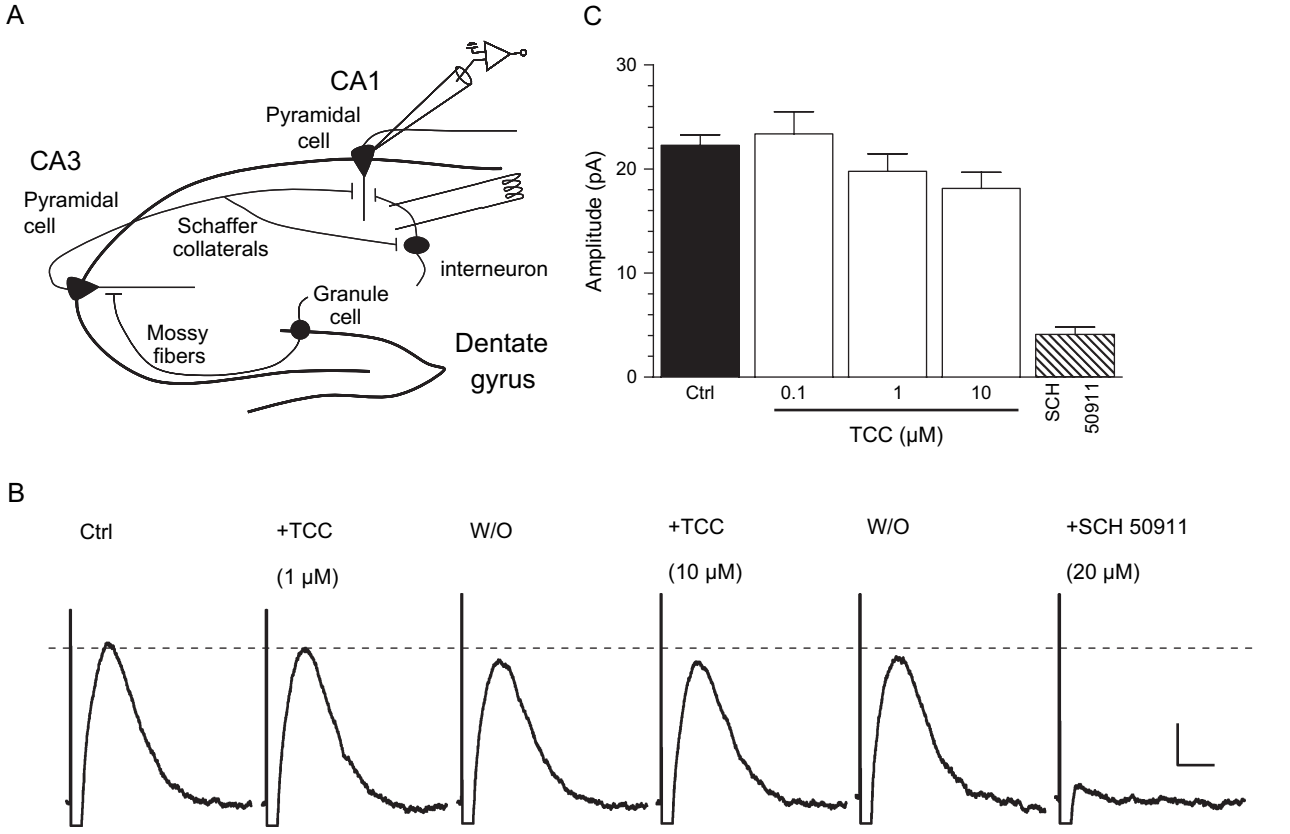

Fig. 6. Role of $GABA_B$Rs in TCC action. (A) Simplified representation of the circuitry of the rat hippocampus relevant to the present study. Stimulation of the axons of interneurons in the stratum radiatum of the CA1 region, close to the recorded CA1 pyramidal neurons, induces massive release of GABA and the activation of postsynaptic $GABA_B$Rs. (B) Representative traces of $GABA_B$R-dependent K^+ currents evoked in a CA1 pyramidal neuron. Membrane potential was held at −50 mV in the presence of kynurenic acid (3 mM) and bicuculline (20 μM). Bath applications of TCC at 1 and 10 μM and of SCH 50911 (20 μM) were separated by washout periods of ≥5 min. Scale bars: current amplitude, 5 pA; time, 200 ms. (C) Summary of the effects of TCC at 0.1 μM ($n = 7$), 1 μM ($n = 5$), and 10 μM ($n = 5$) and of 20 μM SCH 50911 ($n = 7$) on $GABA_B$R-dependent K^+ currents in CA1 pyramidal neurons. Data are the means of the amplitude ± SEM.

>60 mg/kg (Macdonald and Olsen, 1994; Sieghart, 1995). However, injection of non-convulsant doses of PTZ (35 or 45 mg/kg) subsequent to injection with TCC at 15 mg/kg induced seizure activity in ≥80% of treated rats (Table 1).

Finally, we tested the effect of TCC in association with the proconvulsant β-carboline derivate FG 7142 (Little et al., 1984; Macdonald and Olsen, 1994; Sieghart, 1995). As expected, i.p. administration of FG 7142 alone at a dose of 25 mg/kg failed to induce seizures. However, injection of this dose of FG 7142 subsequent to administration of TCC at 15 mg/kg resulted in the development of tonic-clonic seizures and death in ∼70% of treated rats (Table 1).

4. Discussion

Previous studies have suggested that TCC acts as an agonist at $GABA_A$Rs in the central nervous system, and that this action might contribute to the muscle relaxant, analgesic, and local anesthetic properties of this drug (Artusi et al., 2003; Biziere et al., 1981; Janbroers, 1987; Marcel et al., 1990; Perucca et al., 1995; Schousboe, 1999). Whereas the results of our study support the notion that TCC interacts

Table 1
Proconvulsant and convulsant activity of TCC in male rats. TCC (10 to 30 mg/kg, i.p.) was administered either alone or 30 min before injection of PTZ (35 to 55 mg/kg, i.p.) or FG 7142 (25 mg/kg, i.p.)

TCC, (mg/kg)	Other drugs (mg/kg)	Seizures onset (min)	No. of animals with seizures	No. of animals dying
10			0/5 (0%)	0/5 (0%)
15			0/5 (0%)	0/5 (0%)
20		29 ± 2	2/5 (40%)	0/5 (0%)
25		25 ± 1	2/5 (40%)	1/5 (20%)
30		20 ± 5	5/5 (100%)	5/5 (100%)
0	PTZ (35)		0/5 (0%)	0/5 (0%)
15	PTZ (35)	6 ± 1	4/5 (80%)	3/5 (60%)
0	PTZ (45)		0/5 (0%)	0/5 (0%)
10	PTZ (45)	10 ± 1	1/5 (20%)	1/5 (20%)
15	PTZ (45)	4 ± 1	8/9 (88%)	8/9 (88%)
0	PTZ (55)	3 ± 1	4/5 (80%)	0/5 (0%)
15	PTZ (55)	2.0 ± 0.5	5/5 (100%)	4/5 (80%)
0	FG 7142 (25)		0/5 (0%)	0/5 (0%)
15	FG 7142 (25)	24 ± 1	2/3 (67%)	2/3 (67%)

The number of animals affected by seizures or death as well as the time to seizure onset (mean ± SEM) after injection of TCC alone or of PTZ or FG 7142 after TCC were determined.

with $GABA_A$Rs (Balduini et al., 1999, 2001; Biziere et al., 1981; Cimino et al., 1996), they provide direct evidence that TCC inhibits the function of these receptors. Our in vitro and in vivo data, together with previous animal and clinical studies (De Riu et al., 2001; Sechi et al., 2003), thus demonstrate that TCC acts as an effective inhibitor of $GABA_A$R function and as a convulsant. We found evidence that allows to suggest that TCC is a competitive antagonist at $GABA_A$Rs and that the potency of its inhibitory effect may depend on the subunit composition of these receptors. Moreover, we showed that $GABA_B$Rs appear to be largely unaffected by TCC, excluding the possibility that they mediate the myorelaxant effect of this drug. Finally, our results demonstrate that, in addition to acting as a convulsant, even in rats with an intact blood–brain barrier, TCC has a proconvulsant profile at lower doses.

4.1. TCC inhibits the function of synaptic $GABA_A$Rs with a higher potency than it does that of extrasynaptic $GABA_A$Rs

Our electrophysiological data show that TCC reversibly inhibits the function of $GABA_A$Rs in cerebellar Purkinje and granule cells as well as in cultured hippocampal neurons. The inhibitory potency of TCC was greater for evoked phasic synaptic currents recorded from Purkinje cells than for the tonic currents recorded from granule cells. These two types of GABAergic inhibitory currents are mediated by receptors with different subcellular localizations, affinities for GABA, and subunits. The synaptic currents in Purkinje cells are mediated predominantly by $GABA_A$Rs that are composed of α1, β2 or β3, and γ2 subunits and which possess a relatively low affinity for GABA (Pirker et al., 2000; Wisden et al., 1996). In contrast, tonic currents in cerebellar granule cells are mediated by $GABA_A$Rs that contain α6, β2 or β3, and δ subunits and which have a high affinity for GABA (Brickley et al., 1996, 2001; Hamann et al., 2002; Rossi and Hamann, 1998; Rossi et al., 2003; Wall and Usowicz, 1997). Our finding that TCC inhibits the function of synaptic receptors with a greater potency than it does on extrasynaptic receptors may suggest that the former receptor subpopulation, which has a lower affinity for GABA, could represent a more sensitive target for the inhibitory action of TCC. Synaptic $GABA_A$Rs containing α1, β2 or β3, and γ2 subunits exhibit a median effective concentration for activation by GABA that is about 50 times the corresponding value for extrasynaptic $GABA_A$Rs containing α6, β2 or β3, and δ (10 versus 0.2 μM) (Saxena and Macdonald, 1996). TCC also reduced the amplitude of sIPSCs recorded from granule cells in a concentration-dependent manner and slightly reduced the decay time constant of eIPSCs in Purkinje cells.

Our functional results are consistent with previous binding data showing that TCC displaced the specific binding of [^{3}H]GABA in rat cortical synaptic membranes (Balduini et al., 1999). The authors of this previous study hypothesized that TCC interacts differentially with $GABA_A$ receptors that possess different affinities for GABA. The same researchers subsequently found that TCC preferentially inhibited the specific binding of [^{3}H]muscimol in sections of rat brain from regions that express low-affinity $GABA_A$Rs, such as those from the cerebral cortex (Balduini et al., 2001). TCC was not able to displace completely the binding of [^{3}H]muscimol in brain regions enriched in high-affinity $GABA_A$Rs, such as the granule cell layer of the cerebellum. It is likely that these high-affinity $GABA_A$Rs in the granule cell layer of the cerebellum are those that contain α6, β2 or β3, and δ subunits.

The preferential inhibition of synaptic versus extrasynaptic $GABA_A$Rs is also a feature of GABAzine (SR 95531), a competitive $GABA_A$R antagonist (Stell and Mody, 2002; Ueno et al., 1997). On the basis of its ability to inhibit the function of synaptic receptors at lower concentrations than those required to block the function of extrasynaptic receptors, GABAzine is commonly used in experimental settings to isolate pharmacologically the tonic component of GABAergic inhibition.

The profile of TCC as a competitive antagonist at $GABA_A$Rs was confirmed by our observation that the efficacy of TCC with regard to inhibition of GABA-evoked Cl^- currents in pyramidal neurons in culture decreased as the concentration of GABA increased. Similarly, the TCC analog colchicine was previously shown to inhibit currents mediated by human recombinant $GABA_A$Rs containing α1, β2, and γ2L subunits expressed in murine L(tK-) cells and to shift the concentration-response curve for GABA to the right (Weiner et al., 1998).

4.2. TCC does not modulate $GABA_B$R-dependent currents in CA1 pyramidal neurons

CA1 pyramidal neurons express functional postsynaptic $GABA_B$Rs that trigger a K^+ current on activation by GABA released from interneurons. Our electrophysiological and functional data support previous biochemical results suggesting that the myorelaxant effect of TCC is not dependent on an interaction with $GABA_B$Rs. The $GABA_B$R agonist baclofen was thus previously found to be ineffective in displacing the specific binding of [^{3}H]TCC in synaptic cortical membranes (Balduini et al., 1999).

4.3. TCC is a potent proconvulsant and convulsant drug

Our in vivo data show that TCC exerts a convulsant action when administered i.p. in male rats at doses of ≥20 mg/kg (but not at lower doses). These results are in agreement with previous observations showing that TCC was ineffective in inducing seizures in healthy rats at doses up to 12 mg/kg (Sechi et al., 2003). TCC, being a highly hydrophilic molecule, thus appears to have a limited capacity to cross the blood–brain barrier (De Riu et al., 2001; Sechi et al., 2003). We also found that, at doses of 10 to 15 mg/kg, which were ineffective alone in inducing convulsions, TCC acted as a proconvulsant agent when injected in association with non-convulsant doses of PTZ. Furthermore, a non-convulsant dose of TCC (15 mg/kg) potentiated the proconvulsant action of the β-carboline FG 7142. Together, these data demonstrate that TCC is an effective proconvulsant agent that is able to synergistically potentiate

the effects of others antagonists and negative modulators of $GABA_A$Rs.

5. Conclusions

We have shown that TCC is a potent antagonist of $GABA_A$R function. This action is consistent with and may explain the epileptogenic effects of TCC observed under specific experimental and clinical conditions (De Riu et al., 2001; Sechi et al., 2003). In contrast, the mechanisms by which TCC exerts its myorelaxant and analgesic effects remain to be elucidated. Given that, especially in the spinal cord, TCC binds to additional targets, such as glycine receptors (Balduini et al., 1999, 2001), it is likely that this widely used drug induces its therapeutic effects by interacting with various types of neurotransmitter receptor. Indeed, the function of rat glycine receptors expressed in *Xenopus laevis* oocytes appears to be inhibited by TCC at concentration in the low micromolar range (M.P. Mascia et al., unpublished observations).

Acknowledgments

This work was supported by PRIN grant 2004050347 from the Ministry of Instruction, University and Research of Italy and by GIO.I.A. Foundation Pisa, Italy.

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