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European Journal of Pharmacology 559 (2007) 61–64
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Short communication

Inhibitory effects of tutin on glycine receptors in spinal neurons

Jorge Fuentealba [a,c,*], Leonardo Guzmán [a,c], Paula Manríquez-Navarro [a], Claudia Pérez [b,c], Mario Silva [b], José Becerra [b,c], Luis G. Aguayo [a,c]

[a] Department of Physiology, University of Concepcion, Chile
[b] Department of Botany, University of Concepcion, Chile
[c] Patagonian Ecosystems Research Center (CIEP), Chile

Received 21 June 2006; received in revised form 7 December 2006; accepted 14 December 2006
Available online 17 January 2007

Abstract

We studied the effects of tutin, a sesquiterpenoid obtained from *Coriaria ruscifolia subspecie ruscifolia*, a native poisonous Chilean plant, on spinal glycine receptors using patch clamp recordings. In addition, cytosolic Ca^{2+} transients and activation of cAMP response element-binding protein (CREB) were measured in the presence of tutin. Application of tutin (1–1000 μM) inhibited the glycinergic evoked current in a concentration-dependent manner. Moreover, the frequency of spontaneous Ca^{2+} spikes and spontaneous synaptic activity (AMPAergic events) was augmented and correlated with an increase in phosphorylated CREB levels, suggesting an enhancement in neuronal excitability. These results may explain the toxic effects of the plant characterized by seizures and convulsions with subsequent coma and death seen in humans and mice.

Keywords: Spinal neuron; Tutin; Picrotoxin; Synaptic transmission; Strychnine; *Coriaria ruscifolia*

1. Introduction

Extracts from Coriariaceae have been used in Chinese traditional medicine to treat mental disease; however, in some cases seizures were reported. In addition, it was recently described that an extract of *Coriaria* lactone induced strong seizures in rats (Wang et al., 2003). Therefore, this study suggests that the mechanism of action of this extract may be associated to an increment in excitatory neurotransmission in the central nervous system (CNS). *Coriaria ruscifolia subspecie ruscifolia* is native to Chile and is known for its highly poisonous properties, especially in children that ingest its fruits (Hoffmann, 1982). It was previously suggested that the toxic effect of Coriariaceae may be produced by tutin and coriamyrtin, two of its main components (Reyes et al., 1998; Wang et al., 2003). Interestingly, the structures of these compounds have similarities to picrotoxin (Kudo et al., 1984). Therefore, the toxic effects of *C. ruscifolia* (Garcia Martin et al., 1983) might be on $GABA_A$ and glycine receptors (Takeuchi and Takeuchi, 1969). Using extracellular recordings, it was reported that tutin, in the micromolar range, inhibited GABA induced depolarizations in the frog spinal cord (Kudo et al., 1984) suggesting the involvement of $GABA_A$ receptors. It is presently unknown if tutin can interfere with glycine receptors, which are expressed in lower regions of the CNS (Lynch, 2004).

Glycine receptors are pentameric structures composed of α (1–4) and β subunits. Activation of these receptors by glycine induces a rapid increase in Cl^- conductance which is accompanied by a reduction in the excitability of neurons involved in pain, motor coordination and respiratory rhythms (Aguayo et al., 2004; Lynch, 2004). These receptors might also be implicated in seizure generation, since potentiation of glycinergic transmission was shown to block seizures (Seiler and Sarhan, 1984). Therefore, we examined the effect of tutin on spinal glycine receptors in an intent to explain the toxicity of the plant described in mice and humans (Garcia Martin et al., 1983; Wang et al., 2003).

* Corresponding author. Laboratory of Neurophysiology, Department of Physiology, University of Concepcion, PO Box 160-C, Concepción, Chile. Tel.: +56 41 207318; fax: +56 41 245975.
E-mail address: jorgefuentealba@udec.cl (J. Fuentealba).

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doi:10.1016/j.ejphar.2006.12.018

2. Materials and methods

The animals were manipulated according to ethical procedures for animal management as outlined by the Animal Use and Care Committee at the University of Concepcion.

2.1. Tutin isolation

Tutin was isolated from dried leaves of *C. ruscifolia* locally collected. The first extraction was made in methanol and then fractioned with solvents of incremental polarity (from hexane to ethyl acetate). The final purification was made from chloroformic and ethyl acetate portions using SiO_2 column chromatography and crystallization. The purity (>99%) was assayed with ^{1}H-RMN and ^{13}C-RMN as previously reported (Okuda et al., 1987; Perry et al., 2001).

2.2. Cell culture

Mouse (C57BL/J6) spinal cord neurons obtained from five to six embryos (13–14 days) were plated at 300,000 cells/ml into 35-mm tissue culture dishes coated with poly-L-lysine (MW 350 kDa, Sigma Chemical, St. Louis, MO). The neuronal feeding medium consisted of 90% minimal essential medium (GIBCO, Grand Island, NY), 5% heat-inactivated horse serum (GIBCO), 5% fetal bovine serum (GIBCO) and a mixture of nutrient supplements (Aguayo and Pancetti, 1994). Fresh media was replaced every 3 days. Experiments were performed on 14 days *in vitro* neurons.

2.3. Intraperitoneal tutin administration

In vivo experiments were carried out on Wistar male rats (250 g, $n=4$). The small number of animals used for *in vivo* toxicity studies was institutionally justified to reduce excessive suffering according to the triple R principle. Tutin diluted in Ringer's solution to concentrations of 1, 3, 5, and 8 mg/kg at a final volume of 1 ml, was intraperitoneally injected beginning with the lower dose. The time required to elicit toxic responses was evaluated according to the following parameters: 1) immobility: cessation of exploratory capacity, 2) muscle spasm: induction of muscle tremors, 3) seizures: a general convulsive crisis and 4) death: cardio-respiratory collapse.

2.4. Electrophysiological recordings

Voltage-clamp recordings were performed in whole cell configuration and acquired with an Axon 200-B amplifier (Axon Instruments, Union City, CA). Patch-clamp microelectrodes were filled with (in mM): 140 KCl, 10 BAPTA, 10 HEPES (pH 7.4), 4 $MgCl_2$, 0.3 GTP and 2 ATP-Na_2, 300 mOSM. The external solution contained (in mM): 150 NaCl, 5.4 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4) and 10 glucose. The holding potential was fixed at −60 mV, recordings were filtered at 5 kHz with a low pass Bessel filter. The solutions were prepared fresh every day. The recordings were made by applying short (2 s) pulses of glycine (30 μM) every 1 min with a rapid perfusion system. After stabilizing the glycinergic current amplitude, tutin was co-applied and the amplitude of the current to glycine re-evaluated. We applied the full range of tutin concentrations (1–1000 μM) to each neuron in order to obtain a concentration-response curve.

Spontaneous synaptic activity was classified according to the decay time constant (τ) of single synaptic events as follows: AMPAergic $\tau<10$ ms, glycinergic $\tau\sim10$–40 ms, and GABAergic $\tau>40$ ms (van Zundert et al., 2004). Decay time constant, amplitude, frequency and number of events were analyzed with the Minianalysis program (Synaptosoft Inc).

2.5. Ca^{2+} transients

Spontaneous Ca^{2+} transients were detected with Calcium Green® (Molecular Probes). Neurons were incubated with

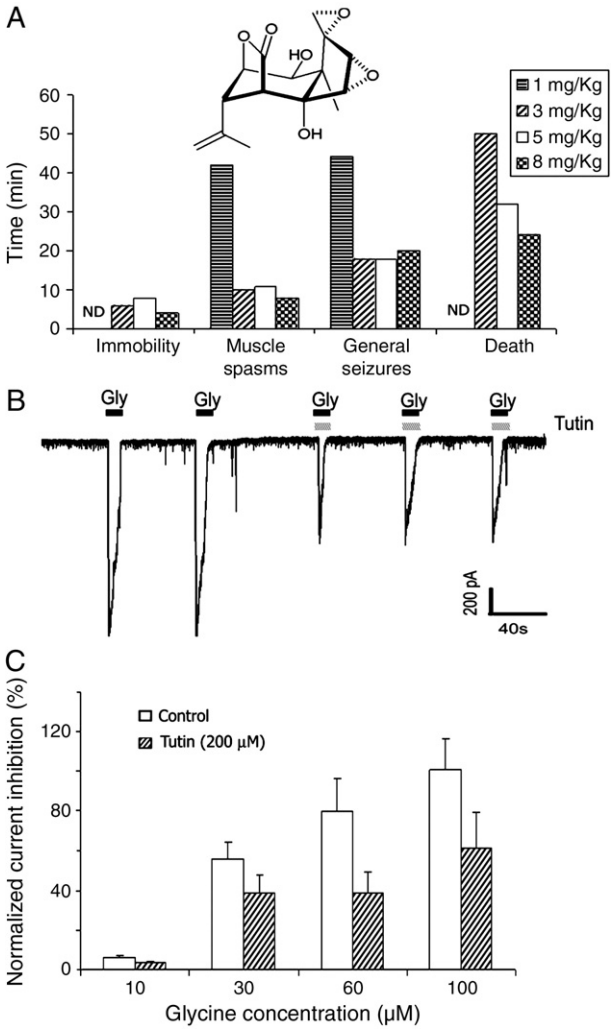

Fig. 1. Effects of tutin on *in vivo* toxicity and evoked glycine current. A: Toxic effects of tutin observed at different doses and times. The inset shows the structure of tutin. B: Current traces evoked by applications of glycine (30 μM) alone and with tutin (200 μM). C: The graph shows the effect of tutin at different concentrations of glycine (10–100 μM). Pulses of glycine were applied for 2 s every 1 min. Tutin was co-applied with glycine. The data shows the normalized current as percentage of control ($n=5$).

3 μM Calcium Green during 30 min and after extensive washing they were placed in a perfusion chamber on a microscope (Nikon, TE 2000). Changes in the cytosolic Ca^{2+} was acquired with a CCD camera (12BIT, SensiCam, PCO) and Lambda 10–2 (Sutter Instruments, USA) interface. Each image was recorded every 1 s and time exposition was 200 ms.

2.6. cAMP response element-binding protein analysis

For cAMP response element-binding protein (CREB) measurements, neurons were treated with different concentrations of tutin for 60 min. The cells were then lysed and the protein concentration of the extracts was measured. Equal amounts of protein (6 μg) were analyzed by polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to nitrocellulose and Ser133 phosphorylated CREB (CREB-P) was identified by Western blot using a specific antibody (Upstate™). The signal of CREB-P was compared to the signal of a load control (Gβ protein) whose expression level was the same in all conditions using the ImageJ software.

2.7. Data analysis

Data are expressed as means±SEM. Statistical comparisons were performed using Student's *t*-test. $p<0.05$ was considered significant.

3. Results

3.1. Animal toxicity

The toxicity of purified tutin (inset Fig. 1A) was examined in rats after intraperitoneal injection. No lethal effects were observed when the lower dose was used (1 mg/kg). As the dose was incremented, the animals showed progressive symptoms of immobility, spasm, seizures and death. The data showed that the time to induce toxicity decreased with increased dosage ($n=4$).

3.2. Effects on whole-cell currents

Short applications of glycine (30 μM) to spinal neurons elicited a Cl^- current that was stable in control condition for at least 20 min and was blocked by 100 nM strychnine (van Zundert et al., 2004). The amplitude of the current was reduced by approximately 50% when tutin (200 μM) was co-applied with glycine (Fig. 1B). This current inhibition was maintained and did not show further increments with repetitive applications. In addition, the inhibition induced by tutin in spinal neurons was only slightly reversible (20±11%) after 3 min of washout with external solution ($n=7$). The effect of tutin was concentration dependent, thus it was reduced by 22±8% with 100 μM and 44±11% with 200 μM ($n=7$). In addition, we found that the effect of tutin was not reversed even using a supramaximal concentration of glycine (100–500 μM, 57±7% of control), suggesting a noncompetitive mechanism (Fig. 1C), similar to picrotoxin (Lynch, 2004). Interestingly, the effect of tutin (200 μM) was not selective for glycine receptors, since we found that it also inhibited the amplitude of the $GABA_A$-induced current to 28±9% of control in the same neurons. On the other hand, the inward current induced by application of glutamate was not significantly altered by tutin (82±7% of control, $n=5$).

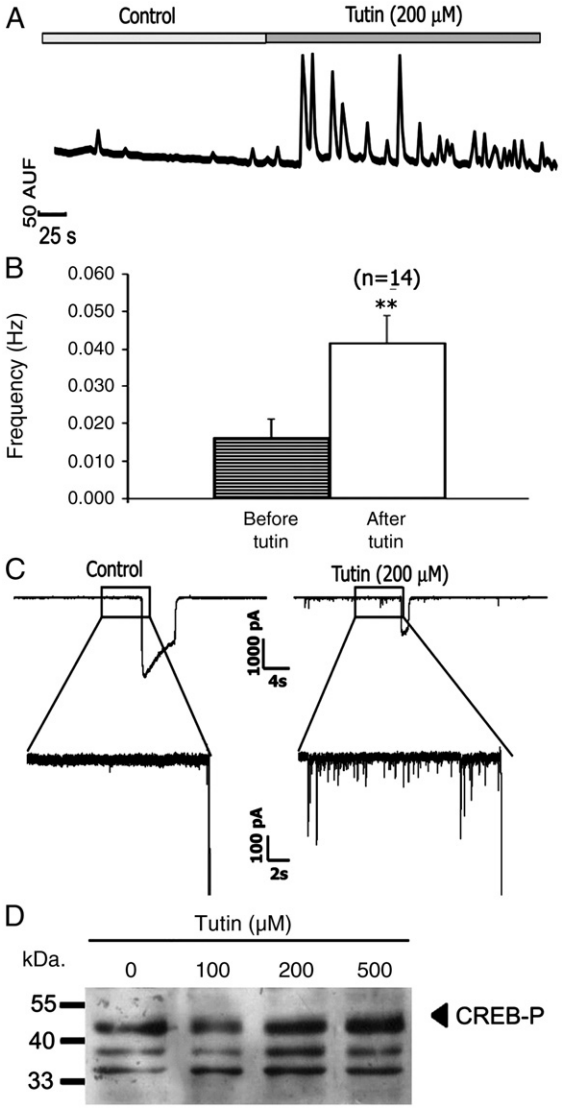

Fig. 2. Effects of tutin on Ca^{2+} transients and phosphorylated-CREB. A: Fluorometric recordings of spontaneous calcium activity in a single neuron. The neurons were incubated with 3 μM Calcium Green® and the images were acquired in the absence and presence of tutin (200 μM) perfused into the bath. The increments are measured over basal fluorescence and expressed as Arbitrary Units of Fluorescence (AUF; $n=5$). B: Frequency (Hz) of spontaneous Ca^{2+} transients before (bar with horizontal lines) and during the perfusion of tutin (white bar; $n=14$; $^{**}p<0.01$). C: Original traces of spontaneous synaptic activity from control and tutin (200 μM) conditions. Note that the evoked current induced by 30 μM glycine was also reduced. D: Western blot analysis of lysates from cells treated with tutin (100–500 μM) and detected with an antibody against phosphorylated-CREB (ser 133) (CREB-P). The position of CREB-P is indicated by the arrowhead ($n=3$).

3.3. Effects on synaptic transmission and calcium transients

We reasoned that if tutin is blocking the inhibitory influence of glycine receptors and GABAergic activities, this should be

reflected by an increase in excitability as observed in spontaneous synaptic activity. Fig. 2A shows that neurons responded with an increase in spontaneous Ca^{2+} transients upon application of tutin (200 μM). Fig. 2B shows that the frequency increased from 0.016±0.005 Hz in control condition to 0.040±0.007 Hz in the presence of tutin (n=14; **p<0.01). These results confirm that tutin augmented overall neuronal network activity. Additionally, with electrophysiological experiments, we found that the sustained perfusion of tutin (200 μM) into the bath caused an increment of 53±14% in overall spontaneous synaptic activity (n=6, p>0.05). Fig. 2C shows the increment of synaptic transmission in the presence of tutin. This enhancement was mainly due to an increase in the number of AMPAergic currents compared to that before its application (20±7 *vs* 47±10%, n=6, *p<0.05).

3.4. cAMP response element-binding protein phosphorylation

The marked increase in neuron activity observed in the presence of tutin might be associated to an increase in Ca^{2+} dependent signal transductions, since it is known that changes in the concentration of this cation affect Ca^{2+}-dependent transcription factors (Dolmetsch et al., 1997, 1998). Therefore, we decided to examine the activation of CREB, a key marker for neuronal activity, which is known to be up regulated during epileptogenic seizure induction and myoclonic status (Dash et al., 1991; Josselyn and Nguyen, 2005). Neurons incubated for 1 h with different concentrations of tutin showed an increase in phosphorylated CREB (Fig. 2D, n=3). For example, the increase in CREB was concentration-dependent with values of 12±1% for 200 μM and 18±2% for 500 μM tutin (n=3).

4. Discussion

In the present study, we analyzed the inhibitory effects of tutin isolated from *C. ruscifolia subspecie ruscifolia*, a native poisonous Chilean shrub, on inhibitory glycine receptors and its impact on neuronal and network excitability in cultured spinal neurons. Tutin was able to block glycine receptors in a concentration-dependent manner and this effect was accompanied by a marked increase in neuronal excitability, reflected by changes in the spontaneous synaptic activity and associated to an increase in the frequency of AMPAergic events. The increase in neuronal activity was likely due to a blockade on both glycine- and $GABA_A$-mediated inhibitory currents by tutin. Interestingly, we found two differences between tutin and corymine, a toxin extracted from the leaves of *Hunteria zeylanica* (Leewanich et al., 1997). First, the effect of tutin was mainly non-competitive in glycine receptors. Second, tutin was more effective in $GABA_A$ receptors than corymine (Leewanich et al., 1997). In addition, we found an augmentation in calcium transients in parallel with an increase in the activated form of CREB, both being indicators of neuronal plasticity and epileptogenic activity (Josselyn and Nguyen, 2005).

In conclusion, tutin appears to play an important role in the toxic effects of the Coriariaceae family by inhibiting glycine receptors in spinal neurons. In addition, it is likely that tutin also induced toxic effects by inhibiting $GABA_A$ receptors.

Acknowledgment

We thank Mrs. L. J. Aguayo and C. Lopez for their technical assistance. This work was supported by the "Programa Bicentenario de Insercion de Postdoctorales en la Academia PBCT Nro. 7" (CONICYT-World Bank) and Proyecto DIUC-Patagonia (University of Concepcion). Patagonian Ecosystems Research Center (CIEP), University of Concepcion, FONDECYT 1020475, 1060368 and Innova BioBio 05B1397L7.

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