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Potentiation and inhibition of glycine receptors by tutin

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ABSTRACT

In the present study we characterized the effects of the South American neurotoxin tutin on recombinant glycine receptors (GlyR) expressed in HEK 293 cells using whole-cell patch-clamp techniques. Tutin induced a concentration-dependent inhibition of α_1 and α_2 homomeric GlyRs, with IC_{50}s of 35 ± 1 and 15 ± 3 μM, respectively. The co-expression of αβ subunits reduced the potency of tutin, thus increasing the IC_{50} to 51 ± 4 and 41 ± 8 μM for $\alpha_1\beta$ and $\alpha_2\beta$ GlyRs, respectively. The inhibitory effect of tutin was competitive, independent of membrane potential and reversible suggesting a pore independent site. On the other hand, low tutin concentrations enhanced the current, which was not synergic with Zn^{2+} or ethanol. A mutation in Lys385 altered ethanol but not tutin sensitivity, suggesting different sites for modulation of α1-containing GlyRs. Our results suggest that tutin affects the GlyR by a mechanism distinct to that of picrotoxin and ethanol, and that the pharmacological profile of tutin exhibits a "Zn-like" behaviour. In conclusion, these results provide information on molecular mechanisms important for understanding the toxic effects of a recently discovered South American neurotoxin.

1. Introduction

Tutin, a picrotoxane molecule, is a poisonous compound recently isolated from *Coriaria ruscifolia* subspecie ruscifolia, a native shrub from the southernmost region of Chile, that is known for having marked neurotoxic properties in humans and that can lead to death (Hoffmann, 1982; Garcia Martin et al., 1983). Extracts from Coriariaceae have been used in Chinese traditional medicine to treat mental disease, with seizures being observed in a few patients (Wang et al., 2003). Interestingly, the structure of tutin has many similarities to picrotoxin (Kudo et al., 1984), and animal toxicity studies showed that rodents experienced muscle spasms, seizures and respiratory paralysis (Fuentealba et al., 2007). We recently characterized the inhibitory effects of tutin, at doses of milligram/Kg, on native glycine and $GABA_A$ receptors in spinal neurons.

Glycine and GABA are main neurotransmitters mediating fast inhibitory neurotransmission (Aguayo et al., 2004; Muller et al., 2008; Lynch, 2009). GlyRs, as well as $GABA_A$Rs, belong to the cys-loop ligand-gated channel (LGIC) superfamily (Cleland, 1996; Cully et al., 1996; Aguayo et al., 2004; Lynch, 2004) and display a pentameric structure containing a central pore permeable to Cl^- (Barnard et al., 1998; Bormann, 2000; Legendre, 2001; Lynch, 2004; Maksay and Biro, 2005; Janssen et al., 2007). GlyRs are competitively inhibited by the convulsant alkaloid strychnine (Akaike and Kaneda, 1989; Betz, 1991; Bechade et al., 1994) with an IC_{50} in the nanomolar range. On the other hand, low concentrations of Zn^{2+} or ethanol, acting at different sites, can induce a positive modulation on GlyR function (Aguayo and Pancetti, 1994; Bloomenthal et al., 1994; Suwa et al., 2001; Miller et al., 2005a; Miller et al., 2005b). Picrotoxin (PTX), on the other hand, was suggested to act as a GlyR blocker through a use-independent manner (Lynch et al., 1995), whereas it appeared to inhibit $GABA_A$Rs through a use-dependent fashion (Newland and Cull-Candy, 1992). Also, it was suggested that PTX can bind to specific sites within the GlyR channel (Wang et al., 2007). On the other hand, the actions of tutin at the receptor level are largely unknown. Therefore, we studied the effect of tutin on recombinant homomeric and heteromeric GlyRs.

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2. Materials and methods

2.1. Tutin isolation

Tutin was isolated from dried leaves of *Coriaria ruscifolia* locally collected (Concepción, Chile) and the purification has been previously described (Fuentealba et al., 2007). In brief, the final purification was made from chloroformic and ethyl acetate portions and the purity (>99%) was assayed with ^{1}H NMR and ^{13}C NMR.

2.2. HEK Cell culture and transfection

HEK 293 cells were cultured using standard methodologies and transfected with lipofectamine 2000 (Invitrogen) in presence of the PCI vector containing the genes for glycine α_1, α_2 and β subunits and GFP. Recordings were done 18–24 h after transfection. When the heteromeric receptors were expressed, a 1:3 (α:β for GlyR) ratio was used to increase the probability of successful co-expression.

2.3. Electrophysiological recordings in HEK cells

Voltage-clamp recordings were performed in the whole-cell configuration of the patch-clamp technique and acquired with an Axon 200-B amplifier (Molecular Devices). Patch-clamp microelectrodes were filled with an internal solution containing (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 and recordings were filtered at 5 kHz with a low pass Bessel filter. The working solutions were prepared daily from the stock. The recordings were made by applying short pulses (1 s) of glycine every 1 min with a perfusion system. After stabilizing the current amplitude, tutin was co-applied with the agonists and the amplitude of the current was measured. We applied a full range of tutin concentrations (0.001–1000 μM) to single cells in order to obtain a concentration–response curve. In these experiments, glycine was applied for 1 s each 1 min, and tutin was co-applied with glycine during the same time interval after control application. The voltage–current relationship was made by measuring the peak current in the presence of glycine alone or co-applied with tutin in the same cell; the holding potential was incremented by 10 mV from −60 to +30 mV each 1 min.

2.4. Data analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed using Student's *t*-test or ANOVA. $P < 0.05$ was considered significant. All the curves were fitted with the Hill equation and the IC_{50}s were calculated using Origin 8.0 software (Origin Lab. Corporation). The data was fit assuming a single inhibitory binding site isotherm, thus excluding all concentrations that produced current potentiation.

3. Results

3.1. Inhibition of homomeric α_1 and α_2 GlyRs by tutin

We first examined the effects produced by tutin (0.001–1000 μM) on α_1 and α_2 homomeric GlyRs using an equipotent concentration of glycine (EC_{50} for each receptor subtype: $\alpha_1 = 27 \pm 3$ and $\alpha_2 = 90 \pm 7$ μM). Fig. 1 shows the effect of several concentrations of tutin on homomeric α_1 receptors (Fig. 1A, and closed circles in Fig. 1B). At concentrations above 1 μM, tutin caused a concentration-dependent inhibition on the amplitude of the glycine current, causing a complete blockade at the highest concentration tested (1000 μM). Interestingly, tutin enhanced the amplitude of the current at lower concentrations, especially in the α1 subunit (30, 1000 nM). The α_2 homomeric receptor, on the other hand, displayed a higher sensitivity to tutin inhibition than α_1 (Fig. 1B, open circles). Analysis of the data showed that the apparent IC_{50} for the inhibition of α_2 monomeric receptors by tutin was 15 ± 3 μM (Table 1, $n = 6$), while the corresponding value for α_1 monomeric receptors was significantly higher at 35 ± 1 μM ($p < 0.05$, $n = 8$). We also found that the extent of inhibition was similar with co-application of glycine and tutin or pre application of tutin (not shown). Using low concentrations of tutin (≤1 μM), we found that it increased the amplitude of the current above the control value in both α_1 ($30 \pm 4\%$, $p < 0.01$) and α_2 ($16 \pm 9\%$, $p < 0.05$, Table 2) subunits. Additional analysis in α1 showed that an

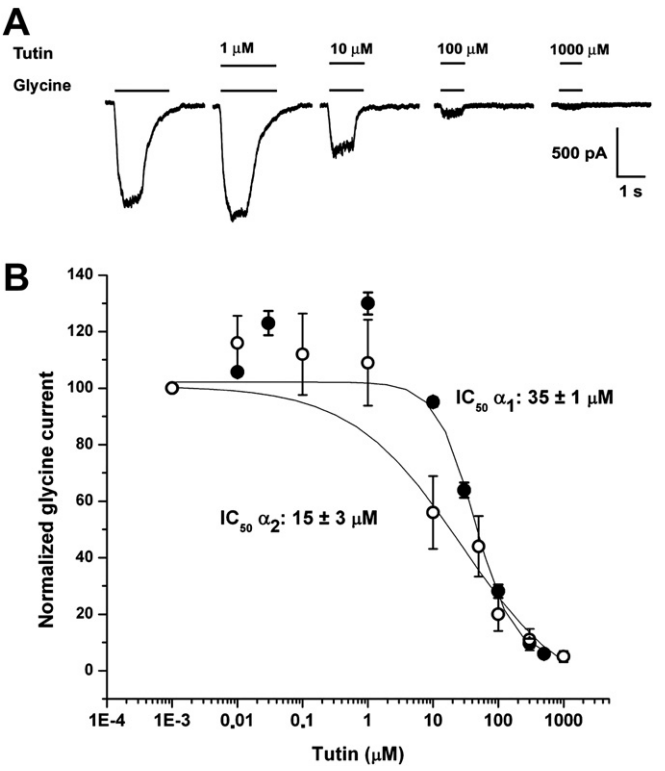

Fig. 1. Inhibitory effects of tutin in homomeric GlyRs. A, the current traces were evoked by glycine (27 μM) on α1 GlyRs alone and in presence of several tutin concentrations. B, the curves are concentration–response relationships for inhibition of the Cl^- current activated with glycine at its EC_{50} ($\alpha_1 = 27 \pm 3$ and $\alpha_2 = 90 \pm 7$ μM). The agonist was co-applied with tutin during 1 s at 1 min intervals at a holding potential of −60 mV. The symbols illustrate the mean ± SEM for α_1 (closed circles, $n = 8$) and α_2 GlyRs (open circles, $n = 5$).

inhibitory concentration (50 μM) shifted the curve towards the right by 56 ± 19% of control (Fig. 2A, closed triangles, $n = 5$), while a potentiating concentration (1 μM) of tutin had the opposite effect shifting the curve to the left by 33 ± 7% (Fig. 2A, closed circles, $n = 8$). Similar behaviour was observed in α2, where 50 μM and 1 μM tutin shifted the curve to the right by 30 ± 5% ($n = 8$) and to the left by 40 ± 2% ($n = 9$), respectively (Fig. 2B).

Application of tutin (200 μM) at the peak of the glycine-evoked current showed that its inhibitory effect developed with a fast onset and that it was highly reversible as shown in the original traces in Fig. 3A obtained from homomeric α2 subunit. For instance, the data show that the glycine current recovered to 86 ± 3.0% in α1 ($n = 5$) and 90 ± 3.0% in α2 ($n = 6$) receptors after tutin application (Fig. 3B). In parallel, the influence of the membrane potential on the

Table 1
Potency of tutin inhibition on different GlyR.

Receptor subtype	Tutin (μM)	PTX (μM)
α_1 GlyR	35 ± 1 ($n = 8$)[a]	9[b]–18[c]
$\alpha_1\beta$ GlyR	51 ± 4 ($n = 8$)[a]	400–1000[b–e]
α_2 GlyR	15 ± 3 ($n = 6$)[a]	3[d]–6[b]
$\alpha_2\beta$ GlyR	41 ± 8 ($n = 7$)[a]	300[b,c]

[a] The data summarizes the values of IC_{50} obtained experimentally in different receptor subtypes. These values were compared with other previously published for blockade with PTX. The IC_{50} values are expressed as mean ± SEM in micromolar concentration. n = number of cells studied.
[b] Pribilla et al., 1992.
[c] Hawthorne and Lynch, 2005.
[d] Aguayo et al., 2004.
[e] Wang-Tilz et al., 2006.