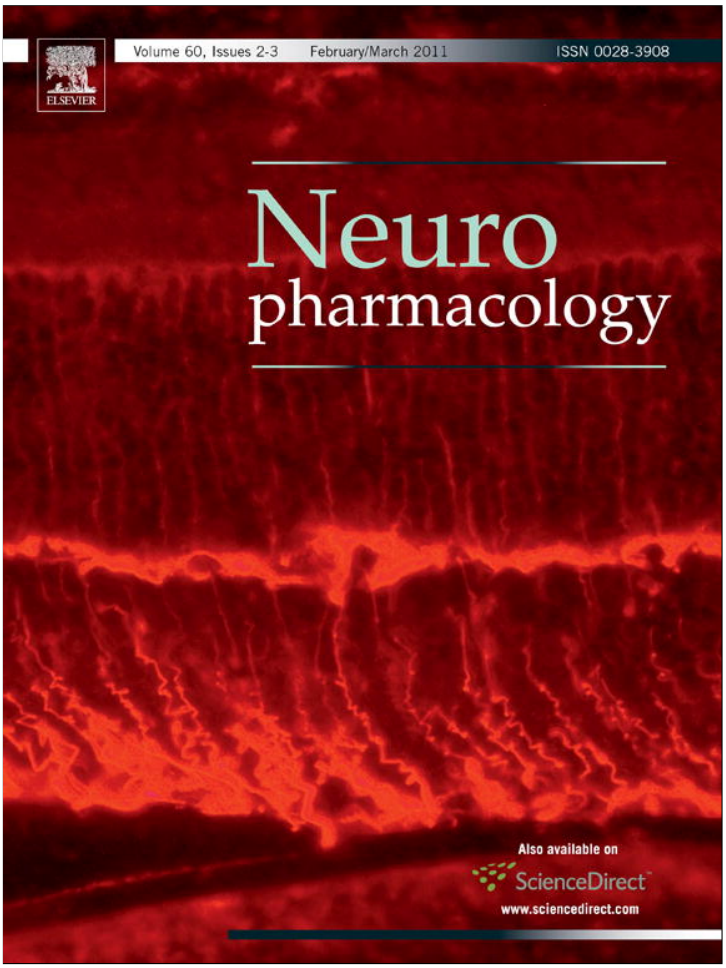

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Potentiation and inhibition of glycine receptors by tutin

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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

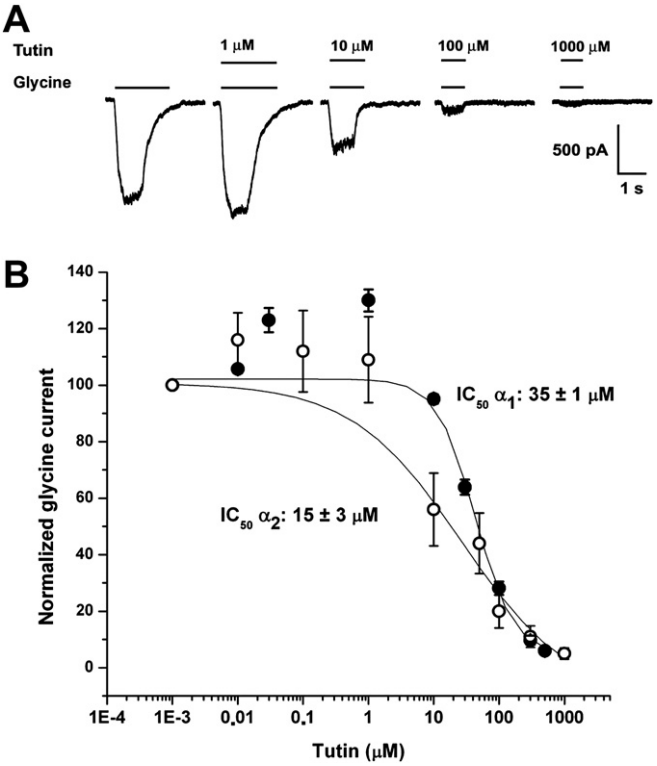

Fig. 1. Inhibitory effects of tutin in homomeric GlyRs. A, the current traces were evoked by glycine (27 μM) on α1 GlyRs alone and in presence of several tutin concentrations. B, the curves are concentration–response relationships for inhibition of the Cl^- current activated with glycine at its EC_{50} ($\alpha_1 = 27 \pm 3$ 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.

Table 2
Potentiation of recombinant GlyR by tutin.

Receptor subtype	Maximal tutin potentiation (%)	Tutin concentration (μM)
α_1 GlyR	30 ± 3.9**[a]	1
$\alpha_1\beta_2$ GlyR	18 ± 5**	0.03
α_2 GlyR	16 ± 9.7*[a]	0.03
$\alpha_2\beta_2$ GlyR	N/O	N/O

[a] The data shows percent of maximal current potentiation induced by low concentrations of tutin on several receptor subtypes. Each value represents the mean ± SEM of current potentiation at the indicated micromolar concentration. n = number of cells studied (**$p < .01$; *$p < 0.05$ over the control). NO: not observed.

inhibition of GlyRs was studied at potentials ranging from −60 to +30 mV. Fig. 4 shows current–voltage relationships in both α1 (panel A) and α2 (panel B) GlyRs in the absence (closed squares) and presence (open squares) of tutin (200 μM, $n = 8$). Analysis of the relative inhibition at different membrane potentials showed that tutin produced the same extent of inhibition at all the voltages examined (Fig. 4, insets).

3.2. Heteromeric β-containing receptors showed reduced sensitivity to tutin

The data depicts that β-containing receptors were less sensitive to inhibition by tutin than their homomeric counterparts (Fig. 5A, Table 1). For example, the inhibition of $\alpha_2\beta$ had an apparent IC_{50} of 41 ± 8 μM (Fig. 5B, open circles, $n = 7$), while the $\alpha_1\beta$ receptor showed an IC_{50} of 51 ± 4 μM (closed circles, $n = 8$). Even at a large concentration (1000 μM), tutin was unable to fully block the current, especially in $\alpha_2\beta$ that showed a resistant component of 19 ± 5% of the current amplitude (Fig. 5B). Finally, applications of low concentrations (0.1 mM) to the $\alpha_1\beta$ conformation showed a potentiation of 18 ± 5% (Fig. 5B, Table 2). In the $\alpha_2\beta$ conformation, on the other hand, potentiation was not observed.

3.3. The effect of tutin was not synergistic to those of PTX, ethanol and Zn^{2+}

Fig. 6A shows the effects of tutin (1 μM) and picrotoxin (0.3 and 3 μM) alone or in combination, at concentrations causing sub-maximal current responses, monitored with propofol, which is a potent allosteric ligand (Yevenes et al., 2008). Data in α1 containing receptors show that picrotoxin caused an even higher diminution of the current than that expected by a simple opposing mechanism with a potentiating concentration of tutin. Additionally, co-application of tutin (1 μM) with a sub-maximal concentration of Zn^{2+} (1 μM) did not produce synergistic effects on α1 (Fig. 6B) or α2 GlyR containing receptors (Fig. 6C). The effects of tutin in two α1 mutants (G254A and R271A), previously described to be resistant to PTX inhibitory actions (Pribilla et al., 1992; Lynch et al., 1995;

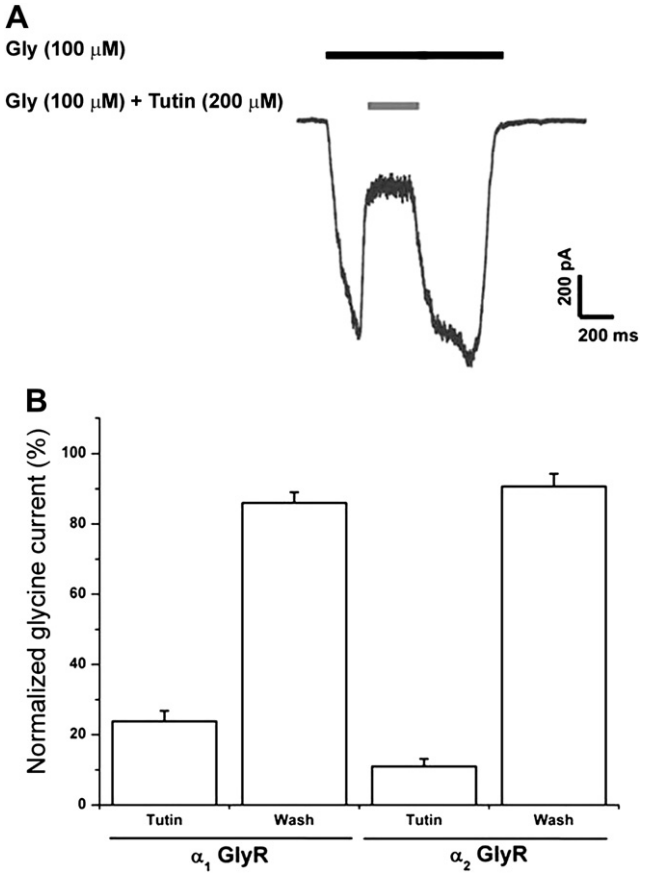

Fig. 3. Reversibility of tutin inhibition on GlyRs. A, the current trace shows onset and recovery from inhibition induced by tutin (200 μM) in α_2 homomeric GlyRs activated with glycine (110 μM). Note the full recovery of the current upon removal of tutin. B, the bars show inhibition and reversibility measured after 1 min washout in homomeric α_1 ($n = 6$) and α_2 ($n = 6$) GlyRs. Each symbol represents the mean ± SEM.

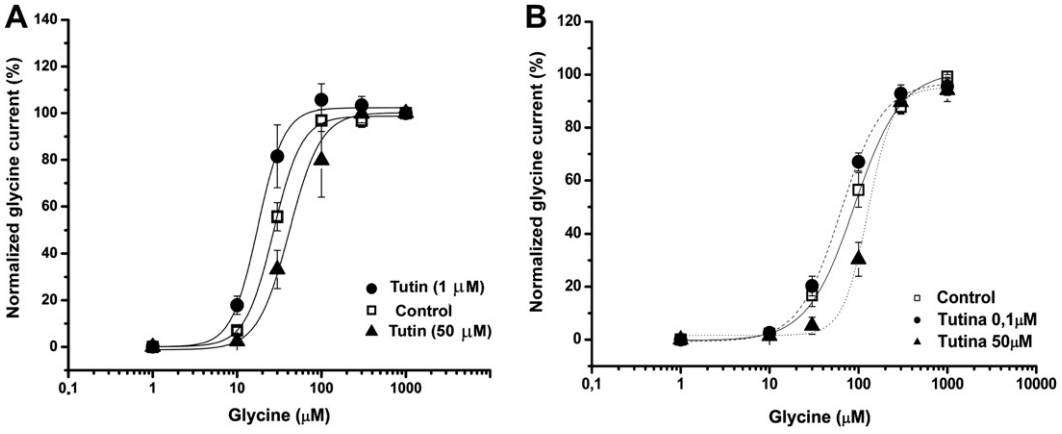

Fig. 2. Effects of tutin concentrations on glycine concentration–response curves. A, the curves represent concentration–response relationships for α_1 subunit activation induced by different glycine concentrations (1–1000 μM) without (open squares, $n = 5$) and co-applied with 1 μM tutin (closed circles, $n = 8$) or 50 μM tutin (closed triangles, $n = 5$). B, similar experimental conditions shown in panel A was applied to the α_2 subunit ($n = 7$). The solid lines were obtained by fitting the data to a single inhibitory site.

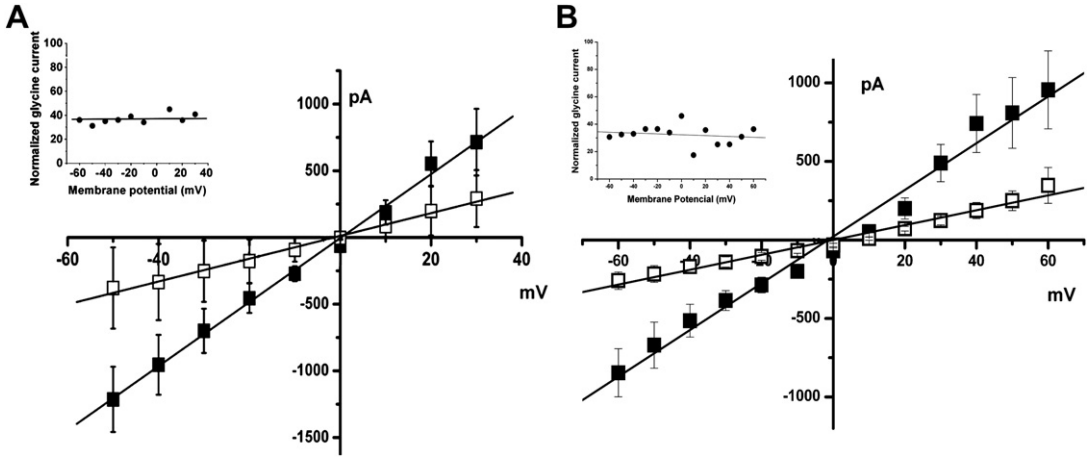

Fig. 4. Voltage-independent inhibition induced by tutin on α_1 and α_2 homomeric GlyRs. The voltage–current relationships were obtained in the absence (closed squares) and presence of tutin (200 μM, open squares) in α_1 subunit (panel A) and α_2 subunits (panel B). The current was activated with 30 μM glycine for the α_1 subunit and 90 μM glycine for the α_2 subunit, at membrane potentials ranging from −60 to +30 mV with steps of 10 mV ($n = 8$). The inset shows the normalized percentage of inhibition produced by tutin at the different potentials tested. The lines are linear fit to the data ($r > 0.99$, $p < 0.0001$).

Hawthorne and Lynch, 2005; Wang et al., 2007), once again show a high complexity. For example, tutin was unable to potentiate the two mutants (Fig. 6D). In addition, while the G254A became more sensitive to tutin inhibition, the R271A was considerably less sensitive to tutin than the wild type GlyR (Fig. 6D).

In addition, co-application of tutin with ethanol showed a lack of synergism in α1 containing receptors (Fig. 7A). Finally, we examined an ethanol resistant mutant of the α1 glycine receptor (Yevenes et al., 2008) and found that tutin was still able to potentiate the K385A mutant receptor suggesting that tutin and ethanol potentiate GlyRs by different mechanisms.

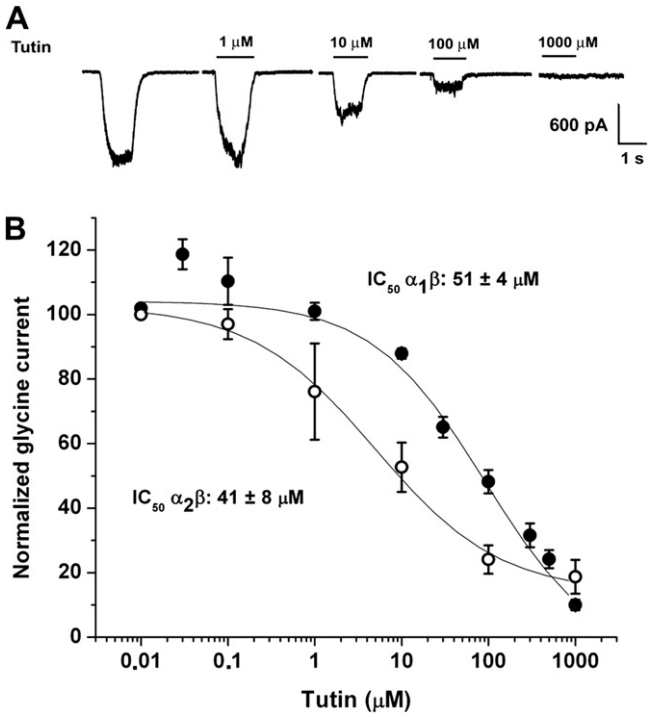

Fig. 5. β subunit co-expression reduced the potency of tutin on GlyRs. A, the current evoked in $\alpha_2\beta$ GlyRs was inhibited by different tutin concentrations. B, the inhibition curves were obtained in the presence of glycine alone (41 μM for $\alpha_1\beta_2$ and 110 μM for $\alpha_2\beta_2$) and different concentrations of tutin. The data was obtained in $\alpha_1\beta_2$ (closed circles, $n = 6$) and $\alpha_2\beta_2$ heteromeric GlyRs (open circles, $n = 5$) Note that 0.03 μM tutin induced a small potentiation of the current in $\alpha_1\beta_2$ GlyRs ($p < 0.01$).

4. Discussion

4.1. Effects of tutin on recombinant GlyR subunits

In the present study we characterized the effects of tutin on recombinant glycine receptor subunits. Additionally, these effects were compared with those of PTX because of their structural similarities (Kudo et al., 1984). For example, it was reported that PTX blocks glycine and $GABA_A$ receptors by complex mechanisms ranging from open channel blockers on $GABA_A$Rs to allosteric competitive antagonists on GlyRs (Wang-Tilz et al., 2006). The idea that PTX binds within the channel pore is supported by data showing that the α1 R271C mutant, a residue thought to be lying in the pore, is less sensitive to PTX blockade (Etter et al., 1999; Hawthorne and Lynch, 2005). Other studies, in addition, have suggested that PTX also binds additional sites in the GlyR (Wang et al., 2007), including sites at the pore-associated TM2 residues 258 and 254 (Dash et al., 1991; Zhang et al., 1995; Burzomato et al., 2004). We found that the mutations in the TM domains caused complex changes in the inhibitory actions of tutin, but they support the conclusion that the sites for receptor potentiation and inhibition are different.

Tutin inhibited homomeric and heteromeric receptors with different potencies with a ranking of $\alpha_2 > \alpha_1 > \alpha_x\beta$ for GlyRs. Although the ranking was similar to that of PTX, there were significant differences in their potency values (see Table 1). Also, tutin showed several unique features, as compared to PTX, when blocking inhibitory receptors. Tutin potentiated these receptors at low concentrations. The blocking effect of tutin on the GlyR was competitive, voltage-independent and readily reversible.

The data indicate that the effect of a low concentration was not additive to either Zn^{2+} or ethanol. This is interesting since despite that these ligands may act at different sites they did not add their effect synergistically (Grudzinska et al., 2005; Yevenes et al., 2008). This can be explained by suggesting that although they attach to distinct positive allosteric sites they cannot enhance channel opening because they affect the pathway between agonist binding and channel gating. Even at a very low concentration of picrotoxin (0.3 μM), the effects of these two ligands were not additive and are

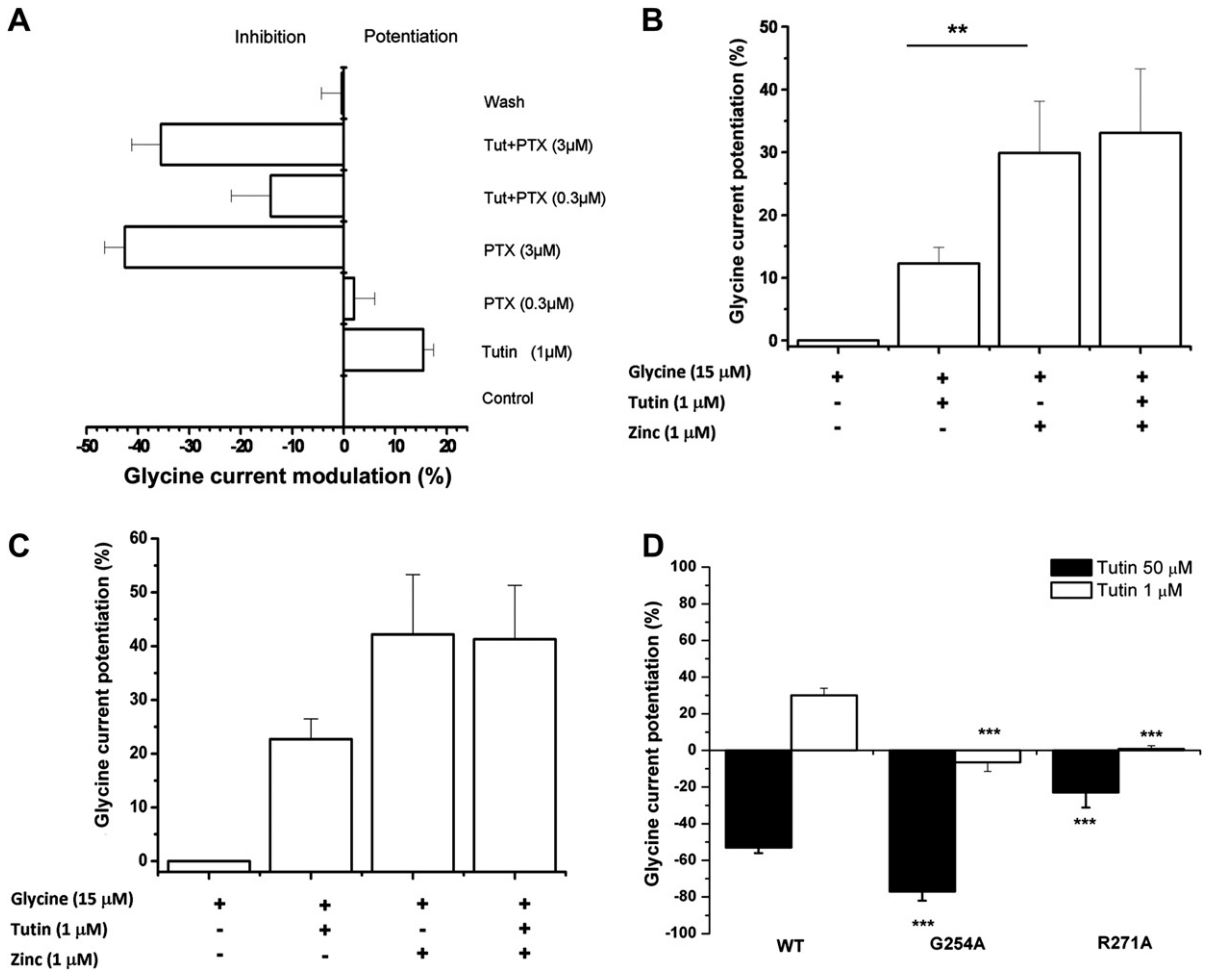

Fig. 6. Effects of tutin alone or co-applied with picrotoxin and Zn^{2+} on the potentiation of GlyRs. A, effects of potentiating tutin concentrations (1 μM) alone and co-applied with picrotoxin at low (0.3 μM) and high (3 μM) concentrations on α_1 subunits ($n = 8$). B, action of tutin (1 μM), Zn^{2+} (1 μM) and combination of both on α_1 GlyRs ($n = 5$, ** $p < 0.01$ tutin vs. Zn^{2+}). C, effects of tutin (1 μM), Zn^{2+} (1 μM) and combination of both on α_2 GlyRs ($n = 6$). D, action of potentiating tutin concentrations (1 μM, white bar) and inhibiting tutin concentrations (50 μM, black bar) on wild type and G254A and R271A α_1 mutant glycine receptors. Each bar represents the mean ± SEM obtained from at least 5 cells ($n = 6$).

suggestive of complex molecular interactions. Thus, the allosteric transduction pathways of glycine and these receptor ligands appear to converge at a common receptor region located prior to channel opening and suggests that they act at a step relevant for extracellular transduction to Cl^- ion flux (Lynch et al., 1995).

4.2. Residues important for agonist binding and modulation

Glycine binding appears to occur at the interface of two adjacent N-terminal domains and involves residues between α and β subunits that are highly conserved (Grudzinska et al., 2005). This is not unique to Cl^- inhibitory channels because $\alpha_4\beta_4$ nACh receptors are also potentiated by interaction of Zn^{2+} with residues located at alternating $\alpha_4\beta_4$ interfaces (Hsiao et al., 2006). Similarly, other studies have demonstrated the presence of residues important for the potentiation of GlyRs by extracellular acting ligands. For example, residues D80, E192, D194 contribute to Cu^{2+} potentiation of α1 GlyRs (Schumann et al., 2009). On the other hand, positions at H107, H109, T112, and T133 are important for receptor blockade by another divalent cation, Zn^{+2} (Miller et al., 2005a). Additionally, key amino acid residues (R271 and R276) in α1 GlyRs are important for PTX binding and provide information on molecular determinants for receptor blockade (Lynch et al., 1995; Hawthorne and Lynch, 2005; Wang et al., 2007). However, this interpretation is complicated because a single amino acid mutation might not be able to significantly alter the binding site for either PTX or tutin and only systematic mutagenesis studies on these regions will be able to confirm the possibility for binding overlap. However, these studies might be complicated by the changes on gating properties associated to these particular sets of mutations in the TM regions. Furthermore, A254 in GlyRs is important in the binding and inhibitory action of PTX suggesting that this residue might play a role in the inhibitory effects of these compounds (Rundstrom et al., 1994). Similarly, residues near positions 254–261 in TM2 are important for PTX binding to β_3 subunits in $GABA_A$Rs (Olsen, 2006). In addition, a threonine located at position 246 of the β subunit appears to be important since mutation of this residue to phenylalanine abolished the picrotoxin sensitivity on $GABA_A$ receptors (Gurley et al., 1995). Tutin, on the other hand, was still able to induce inhibition in receptors with mutations in residues 254 and 271 of TM2 (Fig. 6B). Additionally, the data supports the idea that the binding residues are not within the pore and could be, similar to Cu^{2+} and Zn^{2+}, near the glycine binding site (Miller et al., 2005a).

Finally, the effect of tutin was stronger in α2 than in α1 subunits and this is interesting from a toxicological view point since the receptor configuration changes from α2β to α1β in the developing central system (Legendre, 2001), providing a potential explanation

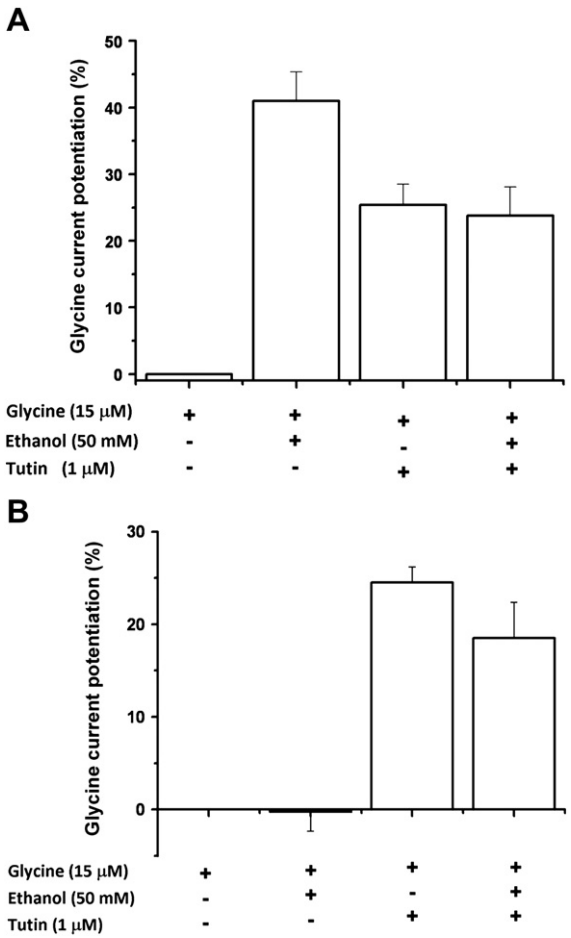

Fig. 7. Effects of tutin applied with ethanol on the potentiation of α_1 GlyRs. A, potentiation of GlyRs by ethanol (50 mM), tutin (1 μM) and both combined. B. Effects of ethanol (50 mM), tutin (1 μM) and co-application in the K385A mutant α1 GlyR. Each bar represents the mean ± SEM obtained from at least 5 cells.

for the larger toxicity of the plant in children (Garcia Martin et al., 1983). Additionally, this study is the first step in finding tutin derivatives that might potentiate inhibitory receptors and therefore be useful as muscle relaxants and pain modulators.

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