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α2 Subunit Specificity of Cyclothiazide Inhibition on Glycine Receptors

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ABSTRACT

In the mammalian cortex, α2 subunit-containing glycine receptors (GlyRs) mediate tonic inhibition, but the precise functional role of this type of GlyRs is difficult to establish because of the lack of subtype-selective antagonist. In this study, we found that cyclothiazide (CTZ), an epileptogenic agent, potently inhibited GlyR-mediated current (I_{Gly}) in cultured rat hippocampal neurons. The inhibition was glycine concentration-dependent, suggesting a competitive mechanism. Note that GlyRs containing the α2 but not α1 or α3 subunits, when being heterologously expressed in human embryonic kidney 293T cells, were inhibited by CTZ, indicating subunit specificity of CTZ action. In addition, the degree of CTZ inhibition on I_{Gly} in rat spinal neurons declined with time in culture, in parallel with a decline of α2 subunit expression, which is known to occur during spinal cord development. Furthermore, site-directed mutagenesis indicates that a single-amino acid threonine at position 59 near the N terminus of the α2 subunit confers the specificity of CTZ action. Thus, CTZ is a potent and selective inhibitor of α2-GlyRs, and threonine at position 59 plays a critical role in the susceptibility of GlyR to CTZ inhibition.

The strychnine-sensitive glycine receptor (GlyR) is a member of cysteine loop family of ligand-gated ion channels (Connolly and Wafford, 2004) and plays important roles in the spinal cord and brain stem (Lynch, 2004; Betz and Laube, 2006). To date, four α subunits (α1, α2, α3, and α4) and one β subunit have been identified (Lynch, 2004). During spinal cord development, there is a switch from α2 subunit to α1 subunit (Watanabe and Akagi, 199; Becker et al., 1988; Aguayo et al., 2004), suggesting a role of α2 subunit-containing GlyRs in neuronal development. This has been supported by two recent studies. First, activation of α2-GlyRs expressed in retinal progenitor cells regulates the number of rod photoreceptors in the developing retina (Young and Cepko, 2004). Second, in the spinal cord, α2-GlyRs regulate interneuron differentiation and the locomotor circuitry in zebrafish (McDearmid et al., 2006). In addition, various GlyR subunits exhibit uneven regional distributions in the adult central nervous system (CNS) (Malosio et al., 1991). In forebrain neurons, the glycine receptor is thought to be mainly a homopentamer of α2 subunits that exert their function extrasynaptically (Brackmann et al., 2004). This implies that, in addition to their significance in development, the variation of GlyRs permitted by the expression of multiple subunits adds a new dimension to information processing capacity of the CNS. For example, in the hippocampus, tonic activation of α2-GlyRs contributes to the modulation of neuronal excitation (Chattipakorn and McMahon, 2003; Song et al., 2006; Zhang et al., 2007), the cross-inhibition of $GABA_A$ receptors (Li and Xu, 2002), and short-term plasticity (Zhang et al., 2006). On the other hand, α3-GlyRs play important roles in modulating spinal inflammatory pain sensitization in the superficial laminae of dorsal horn (Harvey et al., 2004). Evidence for differential roles of GlyRs in neuronal circuits has also emerged from studies on the retina (Ivanova et al., 2006). Therefore, the differences in pharmacology and uneven regional distributions of GlyR subunits suggest that the subunit-specific agonists and antagonists will be very useful for studying GlyR functions in the CNS.

Cyclothiazide (CTZ) was originally developed as a diuretic for the treatment of hypertension (Antlitz and Valle, 1967). In the early 1990s, CTZ was reported to enhance the activity of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of glutamate receptors by suppressing their desensitization (Patneau et al., 1993). Recent evidence indi-

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ABBREVIATIONS: GlyR, glycine receptor; CNS, central nervous system; CTZ, cyclothiazide; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; SDH, spinal dorsal horn; HEK, human embryonic kidney; PTX, picrotoxin; WT, wild type; DIV, day(s) in vitro.

cates that CTZ also exerts an inhibitory effect on $GABA_A$ receptors (Deng and Chen, 2003). Therefore, CTZ interacts oppositely with both AMPA and $GABA_A$ receptors and dramatically elevates the overall neuronal activity in the brain by these two independent mechanisms. More recently, it was found that CTZ is a novel epileptogenic agent that induces robust epileptiform activity in the hippocampus (Qi et al., 2006). In this study, we demonstrated that in addition to AMPA and $GABA_A$ receptors, the inhibitory GlyR is also a target of CTZ. We found that CTZ efficiently reduced I_{Gly} in cultured neurons from embryonic rat hippocampus and spinal cord. Most notably, CTZ exerted an α2 subunit-specific inhibition on GlyRs. These results add a new dimension to the mechanism underlying CTZ-induced epileptogenesis (Qi et al., 2006) and highlight a role of CTZ in the investigation of α2-GlyR function in the CNS.

Materials and Methods

Primary Neuronal Cultures. The care and use of animals in these experiments followed the guidelines and protocols approved by the Care and Use of Animals Committee of the Institute of Neuroscience. Hippocampal and spinal dorsal horn (SDH) neurons from 15- to 18-day-old embryonic Sprague-Dawley rats were isolated by a standard enzyme treatment protocol (Gao et al., 2005). In brief, rat hippocampi and SDH were dissociated in calcium-free saline with sucrose (20 mM) and plated (1–5 $\times$ 10^5 cell/ml) on poly-D-lysine (Sigma-Aldrich, St. Louis, MO)-coated cover glasses. The neurons were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) with L-glutamine plus 10% fetal bovine serum (Invitrogen) and 10% F12 Nutrient mixtures (Invitrogen). Neurobasal medium (1.5 ml; Invitrogen) with 2% B27 serum-free supplements (Invitrogen) was replaced every 3 to 4 days. Treatment with 5-fluoro-5′-deoxyuridine (20 μg/ml; Sigma-Aldrich) on the 3rd day after plating was used to block cell division of non-neuronal cells, which helped to stabilize the cell population. The cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere. Cells were used for electrophysiological recording 5 to 21 days after plating.

Mutagenesis. Mutations of receptor cDNA were performed using a commercially available QuikChange site-directed mutagenesis kit (Stratagene, Shanghai, China) with commercially produced mutagenic primers (Invitrogen). All mutants were verified by DNA sequencing (Invitrogen).

Expression of Recombinant GlyRs. The human α1 and β subunit cDNA were obtained from Dr. Yu-tian Wang (University of British Columbia, Vancouver, BC, Canada). The human α2 subunit cDNA was kindly provided by Dr. Heinrich Betz (Department of Neurochemistry, Max Planck Institute for Brain Research, Frankfurt, Germany). The rat α3 subunit cDNA was donated by Dr. Jochen Meier (Department of Developmental Physiology, Johannes-Mueller Center of Physiology, Charite University Medicine, Berlin, Germany). In brief, HEK293T cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and 100 units/ml penicillin/streptomycin (all from Invitrogen). Transient transfection of HEK293T cells was carried out using calcium phosphate precipitation protocol. Cotransfection with a green fluorescent protein expression vector, pEGFP-N1, was used to enable identification of transfected cells for patch clamping by monitoring green fluorescent protein fluorescence in some experiments. When cotransfecting the GlyR α and β subunits, their respective cDNAs were combined in a ratio of 1:3 to ensure the formation of functional heteroligomers. After exposure to transfection solution for 8 h, cells were washed twice with the culture medium. Electrophysiological recording was performed 24 to 48 h after calcium phosphate treatment.

Electrophysiological Recordings. The cells were observed using a fluorescent microscope, and currents were measured by the conventional whole-cell patch-recording configuration under voltage-clamp conditions. The cells were perfused by the standard external solution contained: 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, with the pH adjusted to 7.3 with Tris base. The osmolarity of all bath solutions was adjusted to 325 to 330 mOsM with sucrose (3300; Advanced Instruments, Norwood, MA). Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830; Narishige, Tokyo, Japan). The resistance of pipettes was 3 to 5 MΩ when filled with the patch pipette solution, which contained: 120 mM KCl, 30 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM EGTA, 2 mM Mg-ATP, and 10 mM HEPES, with the pH adjusted to 7.2 with Tris base. When the I-V relationships for I_{Gly} were examined, 300 nM tetrodotoxin and 100 μM $CdCl_2$ were added to the standard external solution, and K^+ was replaced with Cs^+ in the pipette solution. Membrane currents were measured using a patch-clamp amplifier (Axon 200B; Molecular Devices, Sunnyvale, CA), filtered with a cut-off frequency of 2 kHz, sampled at a rate of 100 kHz, and analyzed using a Digidata 1320A interface and a personal computer with Clampex and Clampfit software (version 9.0.1; Molecular Devices). Unless otherwise noted, the membrane potential was held at -60 mV throughout the experiment. All the experiments were carried out at room temperature (22–25°C). The average access resistances for neurons and HEK293T cells used in the experiments are all approximately 10 MΩ. The average whole-cell capacitance for the hippocampal neurons, the spinal cord neurons, and HEK293T cells is approximately 25, 20, and 30 pF, respectively.

Drugs. All drugs were purchased from Sigma-Aldrich. Picrotoxin (PTX) and CTZ were initially dissolved as concentrated stocks solutions in dimethyl sulfoxide and subsequently diluted to the desired concentration in standard external solution. The final dimethyl sulfoxide concentration was lower than 0.1%. Other drugs were first dissolved in ion-free water and then diluted to the final concentrations in the standard external solution just before use or dissolved directly in the standard external solution. Drugs were applied using a rapid application technique termed the "Y-tube" method throughout the experiments. This system allows a complete exchange of external solution surrounding a neuron within 20 ms (Murase et al., 1990). Throughout the experiment, the bath was superfused continuously with the standard external solution.

Data Analysis. The software Clampfit 9.0.1 (Molecular Devices) was used for data analysis. The continuous theoretical curves for concentration-response relationships of glycine in the presence or absence of CTZ were drawn according to a modified Michaelis-Menten equation by the method of least squares (the Newton-Raphson method) after normalizing the amplitude of the response: $I = I_{max} C^{nH}/(C^{nH} + EC_{50}{}^{nH})$, where I is the normalized value of the current, I_{max} is the maximal response, C is the drug concentration, EC_{50} is the agonist concentration producing a half-maximal response, and n_H is the apparent Hill coefficient. The curve for the effect of CTZ on I_{Gly} was fitted by the following equation: $I = I_{max}$ $(IC_{50})^{nH}$ / $(C^{nH} + IC_{50}{}^{nH})$, where IC_{50} represents the antagonist concentration producing a half-maximal inhibitory effect, and the others are the same as described above. All data were calculated as the mean $\pm$ S.E.M. When quantification analysis was made, statistical comparison was carried out using Student's t test for the comparison of two groups. Statistically significant differences were assumed as $P < 0.05$ for all data. P and n represent the value of significance and the number of neurons, respectively.

Results

CTZ Effects on Cultured Rat Hippocampal Neurons. Glycine and taurine are the major endogenous ligands of GlyRs in mammalian CNS (Mori et al., 2002); therefore, we

first examined the effects of CTZ on both glycine- and taurine-evoked currents (I_{Gly} and I_{Tau}, respectively) in cultured rat hippocampal neurons. At a holding potential of −60 mV under the whole-cell voltage clamp mode, bath application of glycine (100 μM) and taurine (500 μM in the presence of 10 μM bicuculline for blocking $GABA_A$ receptor) evoked inward currents in all tested neurons under our experimental conditions (161 and 153 mM Cl^- in the external and internal solutions, respectively) (Fig. 1A). These currents were strychnine-sensitive (data not shown). Addition of CTZ (100 μM) resulted in a rapid reduction of the current amplitude (Fig. 1, A and B), although CTZ itself produced no detectable responses. The effect was reversible because both I_{Gly} and I_{Tau} were recovered after 2 min of wash-off. The concentration dependence of CTZ inhibition was investigated with 30 and 100 μM glycine. Figure 1C shows that CTZ caused a concentration-dependent reduction of I_{Gly}, and the IC_{50} value of CTZ for the current induced by 30 μM glycine is 22.2 ± 4.2 μM and significantly increased to 95.7 ± 2.0 μM for that induced by 100 μM glycine. This significant alteration of the CTZ concentration-response relationship by glycine concentration suggests the competitive interaction between glycine and CTZ.

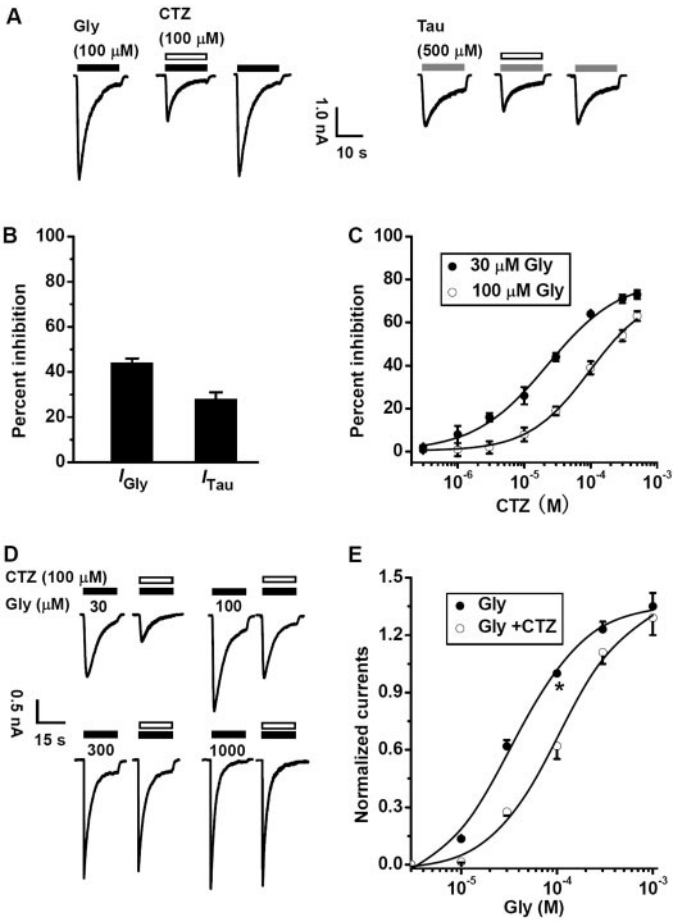

Fig. 1. CTZ reduces both glycine- and taurine-evoked currents in cultured rat hippocampal neurons. A, representative recordings showing 100 μM Gly-evoked current (I_{Gly}) and 500 μM Tau-evoked current (I_{Tau}) in the absence or presence of 100 μM CTZ. Note the rapid recovery of responses after washing out of CTZ. B, histograms show the percentage inhibition of I_{Gly} and I_{Tau} by 100 μM CTZ (n = 8–13). C, concentration-response relationship for CTZ-induced reduction of current activated by 30 (●) or 100 (○) μM glycine. All responses were normalized to the peak I_{Gly} induced by 100 μM glycine alone (n = 8–14). Continuous line was the result of fitting to the Hill equation (see *Materials and Methods*). D, typical recordings obtained from a single neuron illustrating the effect of 100 μM CTZ on I_{Gly} activated by various concentrations of glycine. E, concentration-response curves for Gly alone (●) or glycine plus 100 μM CTZ (○) (n = 6–8). All responses were normalized to the peak current evoked by 100 μM glycine alone (*). Note the rightward shift of the curve in the presence of CTZ.

To elucidate whether CTZ interferes with glycine binding to the GlyR, we recorded I_{Gly} in the absence or presence of CTZ. We found that the inhibition of CTZ depended on glycine concentrations (Fig. 1D). As shown in Fig. 1E, the EC_{50} of I_{Gly} without CTZ was 34.1 ± 6.5 μM, and in the presence of 100 μM CTZ, this was increased to 105.4 ± 19.9 μM. In addition, CTZ did not significantly alter the Hill coefficient of I_{Gly} (without CTZ, 1.1 ± 0.2; with CTZ, 1.2 ± 0.3). The apparent increase of the EC_{50} by CTZ further supports a competitive nature of its inhibition.

To investigate the voltage sensitivity of CTZ inhibition, we measured the I-V relationships of I_{Gly}. CTZ equally inhibited I_{Gly} at either positive or negative voltages, as revealed by the similar reduction of I_{Gly} at −30 mV (47.9 ± 5.0%) and +30 mV (45.2 ± 4.0%), respectively. In addition, CTZ did not significantly alter the reversal potential of I_{Gly}. The potentials (without CTZ, 1.8 ± 1.4; with CTZ, 1.5 ± 1.5) were all close to the theoretical Cl^- equilibrium potential (E_{Cl}) of 1.3 mV calculated from the given extra- and intracellular Cl^- concentrations with the Nernst equation. Therefore, these results indicate that CTZ inhibition of I_{Gly} is voltage independence.

Use Independence of CTZ Inhibition. The concentration dependence of CTZ effect suggests a competitive manner of CTZ inhibition. We tested this hypothesis further by comparing the inhibition of I_{Gly} with four different CTZ application protocols. As shown in Fig. 2, A and B, pretreatment of CTZ followed by coapplication of CTZ and glycine produced the most marked inhibition, although prolonged pretreatment of CTZ did not further the effect. However, the inhibition caused by the sequential application of CTZ and glycine (Fig. 2, Ab and B) was indistinguishable from that obtained with coapplication (Fig. 2, Ac and B). That CTZ interacts with GlyR before glycine application suggests that CTZ inhibition of I_{Gly} is independent of channel opening, favoring the idea that CTZ competes with the agonist at the GlyR.

As shown in Fig. 2C, CTZ pulses rapidly reduced I_{Gly} in the continuous presence of glycine, consistent with a direct interaction of CTZ with GlyRs, without involvement of any intracellular signaling pathways. This experiment also showed that repeated CTZ application inhibited I_{Gly} to a similar extent, indicating that the inhibition is use-independent.

Additive Effect of Picrotoxin and CTZ. To make sure that CTZ really inhibits GlyRs independent of channel opening, we further examined the mutual effect of CTZ and PTX, a representative open-channel blocker of GlyR and $GABA_A$ receptor chloride channels (Yoon et al., 1993; Wang et al., 2006), on I_{Gly}. As shown in Fig. 3, the current induced by 100 μM glycine was inhibited to 79 ± 3 and 50 ± 3% of control by PTX (100 μM) and CTZ (300 μM, the saturating concentration), respectively. When PTX (100 μM) and CTZ (300 μM) were applied together, the I_{Gly} was further depressed to 29 ± 4% of control. In addition, the I_{Gly} was inhibited to 22 ± 2% of control by the maximally efficacious concentration of PTX (1 mM) and was further inhibited to 9 ± 2% of control by

adding 100 μM CTZ. If PTX and CTZ share a common mechanism of inhibition, the maximal inhibition induced by the maximally efficacious concentration of PTX should occlude further inhibition of the glycine response by CTZ. Thus, the additive effect of CTZ and PTX inhibition suggests that the two compounds may block different populations of GlyRs or exert their effect through distinct binding sites on the GlyR.

CTZ Inhibition Was α2 Subunit-Specific. Previous studies have shown that hippocampal neurons express multiple GlyR isoforms (Malosio et al., 1991). To determine the subunit specificity of CTZ inhibition, we examined the CTZ effect in HEK293T cells expressing the α1, α2, and α3 subunits of the GlyR, either alone or together with the β subunit. The EC_{50} and n_H values for each of the six receptor subtypes are summarized in Table 1. As shown in Fig. 4A, the currents activated by 100 μM glycine were recorded in HEK293T cells expressing different homomeric (α1, α2, or α3) and heteromeric ($\alpha1\beta$, $\alpha2\beta$, or $\alpha3\beta$) GlyRs. Furthermore, CTZ inhibited I_{Gly} mediated by homomeric α2- and heteromeric $\alpha2\beta$-GlyRs in a concentration-dependent manner (Fig. 4B). To further compare the difference of CTZ modulation on α1, α2, and α3 GlyR, we test the effects of CTZ on I_{Gly} induced by an equally efficacious concentration of glycine (EC_{70}, 50 μM for α1-GlyR, 100 μM for α2-GlyR, and 150 μM for α1-GlyR) in these homomeric GlyR. In contrast to α2-containing GlyRs, I_{Gly} induced by EC_{70} glycine concentration in α1- and α3-GlyRs were essentially unaffected by CTZ (Fig. 4C). Coexpressing α and β subunits in HEK293T cells with an excess of β subunit leads to the near exclusive formation of functional $\alpha\beta$ heteromers (Pribilla et al., 1992; Bormann et al., 1993). The characteristic reduction in PTX sensitivity was used to test the successful incorporation of β subunit to functional receptors. The IC_{50} value of PTX for homomeric α1, α2, and α3 GlyR was 8.2 $\pm$ 1.4, 3.6 $\pm$ 0.9, and 10.6 $\pm$ 1.9 μM, respectively. After incorporation of the β subunit, the IC_{50} value of PTX was increased to 232 $\pm$ 25, 38.3 $\pm$ 3.8, and 218 $\pm$ 32 μM, respectively. Thus, the significant reduction of

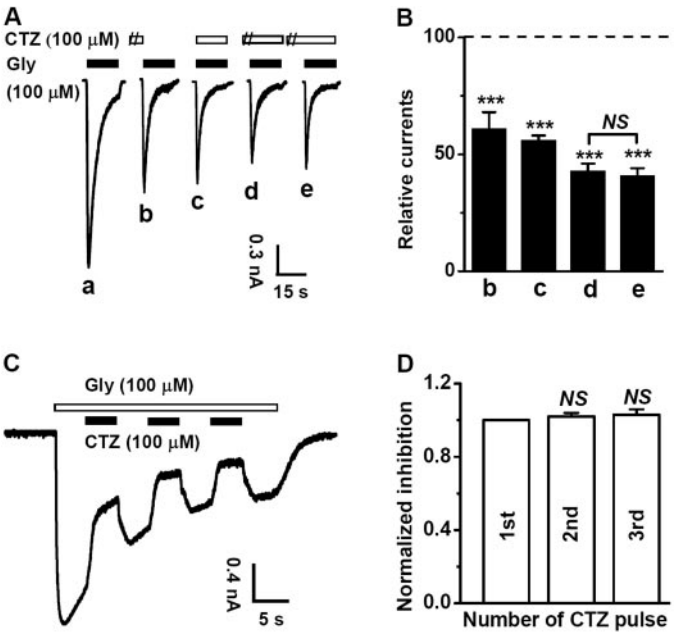

Fig. 2. Effects of CTZ on I_{Gly} with different drug application protocols. A, sample recordings illustrating CTZ inhibitory effect on I_{Gly} with four different CTZ application protocols. A, a, current evoked by glycine alone; A, b, sequential application of CTZ and glycine. CTZ was preapplied for 10 s. A, c, coapplication of CTZ and glycine. A, d, 10-s pretreatment of CTZ followed by coapplication of CTZ and glycine. A, e, 60-s pretreatment of CTZ followed by coapplication of CTZ and glycine. B, histograms showing relative I_{Gly} with four different CTZ application as indicated in A. ***, $P < 0.001$, compared with the currents induced by 100 μM glycine alone (dashed line). NS, no significant difference. $n = 6$. C, three repeated CTZ pulses during continuous application of glycine reduced I_{Gly} to a similar extent, suggesting a use-independent manner of inhibition. D, summary data showing normalized inhibition of I_{Gly} by three separate CTZ pulses. NS, no significant difference, compared with the inhibition induced by the first pulse of CTZ. $n = 5$.

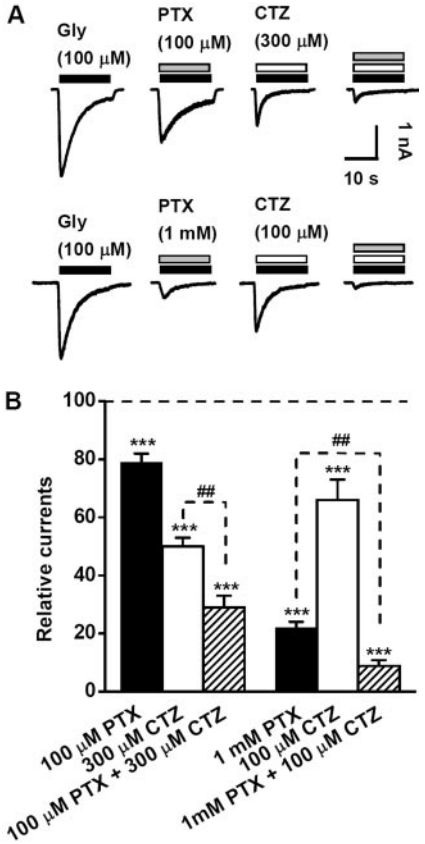

Fig. 3. Additive inhibition of CTZ and PTX on I_{Gly}. A, representative traces showing I_{Gly} in the absence or presence of various concentrations of CTZ and PTX. The maximally efficacious concentration for PTX used in the experiments is 1 mM. B, relative I_{Gly} in the presence of various concentrations of PTX, CTZ, or PTX plus CTZ. ***, $P < 0.001$, compared with the currents induced by 100 μM glycine alone (dashed line). ##, $P < 0.01$, compared with the currents induced by PTX plus CTZ. $n = 7$.

TABLE 1
Functional properties of GlyRs used in this study

GlyR	EC_{50}	n_H	n
	μM		
α1	26.7 $\pm$ 3.7	1.6 $\pm$ 0.3	6
α2	60.2 $\pm$ 3.4	1.8 $\pm$ 0.1	9
α3	86.1 $\pm$ 1.8	1.7 $\pm$ 0.6	6
$\alpha1\beta$	23.9 $\pm$ 3.1	1.9 $\pm$ 0.1	5
$\alpha2\beta$	85.1 $\pm$ 3.6	2.4 $\pm$ 0.3	5
$\alpha3\beta$	63.5 $\pm$ 3.4	1.6 $\pm$ 0.2	5
$\alpha1^{A52T}$	61.3 $\pm$ 4.4	1.8 $\pm$ 0.1	6
$\alpha3^{A52T}$	105.1 $\pm$ 6.9	1.9 $\pm$ 0.1	5
$\alpha2^{T59A}$	48.1 $\pm$ 2.6	1.8 $\pm$ 0.1	8
$\alpha2^{S78P}$	90.4 $\pm$ 1.4	1.4 $\pm$ 0.3	5
$\alpha2^{D117A}$	26.0 $\pm$ 1.6	2.1 $\pm$ 0.3	5
$\alpha2^{T160I}$	100.6 $\pm$ 8.0	2.1 $\pm$ 0.4	5
$\alpha2^{G203R}$	107.1 $\pm$ 12.0	1.7 $\pm$ 0.3	5

PTX sensitivity confirmed that heteromeric GlyRs were functionally expressed in HEK293T cells. We found that the CTZ sensitivity of $\alpha2\beta$ heteromers (IC_{50} = 76 ± 37 μM, n = 5–7) was indistinguishable from that of α2 homomers (IC_{50} = 51 ± 21 μM, n = 5–10).

Threonine at Position 59 Conferred the Specificity of CTZ Inhibition on α2-GlyR. Based on the competitive nature of CTZ inhibition, we hypothesized that the binding site of CTZ was close to the glycine binding domain on GlyRs. By comparing the properties of the extracellular amino acid residues of three GlyR α subunits through sequence alignment, we found that the following residues, Thr59, Ser78, Asp117, Thr160, Leu178, Ser179, and Gly203 in the α2 subunit, differ from the corresponding sites in α1 and α3 subunits. CTZ may bind to these residues and then produce competitive inhibition on α2-GlyRs. To explore which residues may be involved in the CTZ inhibition of α2-GlyRs, the effect of CTZ on I_{Gly} mediated by various mutated forms of homomeric α2-GlyRs with amino acid replacement (T59A, S78P, D117A, T160I, L178Q, S179D, or G203R) was examined. The EC_{50} and n_H values for each of the mutants are summarized in Table 1. We compared the percentage inhibition of CTZ on I_{Gly} in HEK293T cells expressing wild-type (WT) α2- and those carrying α2-GlyR mutants. As shown in Fig. 5C, the glycine EC_{50} value for WT α2-GlyR was 60.1 ± 3.4 μM, and it was decreased to 48.1 ± 2.5 μM by the T59A mutation, suggesting enhanced glycine sensitivity. The glycine EC_{70} value for WT α2-GlyR is 100 μM, which we mainly used for activating GlyR in other experiments, whereas the equally efficacious concentration for mutant α2 (T59A)-GlyR is 80 μM. With this equally efficacious concentration, we compared the CTZ effects on I_{Gly} mediated by WT α2-GlyR and α2 (T59A) mutant. We found that in cells expressing the mutant α2 (T59A) subunit, the percentage inhibition of I_{Gly} was significantly reduced (Fig. 5D). The threshold concentration for statistically significant CTZ inhibition was 10 μM for WT α2-GlyR and 300 μM for the mutated α2-GlyRs (T59A), respectively. Because of the limited solubility of CTZ, no concentration of CTZ greater than 300 μM was used in this study. The percentage inhibition of CTZ (100 μM) on the current induced by 100 μM glycine in α2-GlyR with single mutation of S78P, D117A, T160I, and G203R was 35.6 ± 5.3, 32.7 ± 4.8, 42.1 ± 6.3, and 43.6 ± 7.2%, respectively. Therefore, the effects of CTZ inhibition were not significantly affected by single mutations of S78P, D117A, T160I, and G203R. Because no functional GlyR was obtained by the double mutation of L178Q and S179D, the effects of Leu178 and Ser179 on CTZ inhibition of GlyR were not examined.

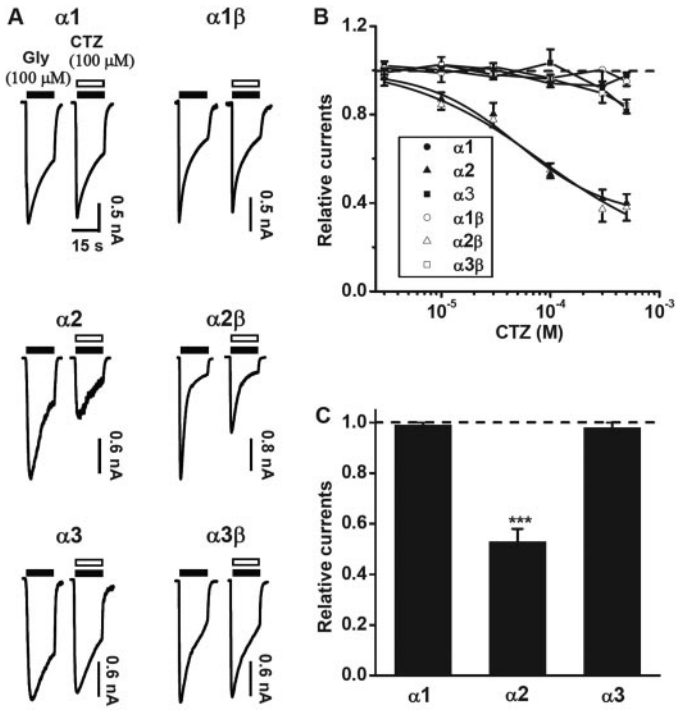

Fig. 4. Subunit specificity of CTZ inhibition of GlyRs. A, representative traces showing the effect of CTZ (100 μM) on I_{Gly} mediated by homomeric α1-, α2-, α3-, and heteromeric α1β-, α2β-, and α3β-GlyRs expressed in HEK293T cells. B, concentration-response relationship of CTZ inhibition on various GlyR subtypes. Each point represents mean ± S.E.M. normalized to the corresponding peak I_{Gly} (dashed line) before CTZ application (n = 5–10). C, histograms showing the relative I_{Gly} activated by EC_{70} glycine concentration (50 μM for α1-GlyR, 100 μM for α2-GlyR, and 150 μM for α3-GlyR) in the presence of CTZ in HEK293T cells expressed with homomeric α1-, α2-, or α3-GlyRs. ***, $P < 0.001$, compared with I_{Gly} before CTZ application (dashed line). n = 6.

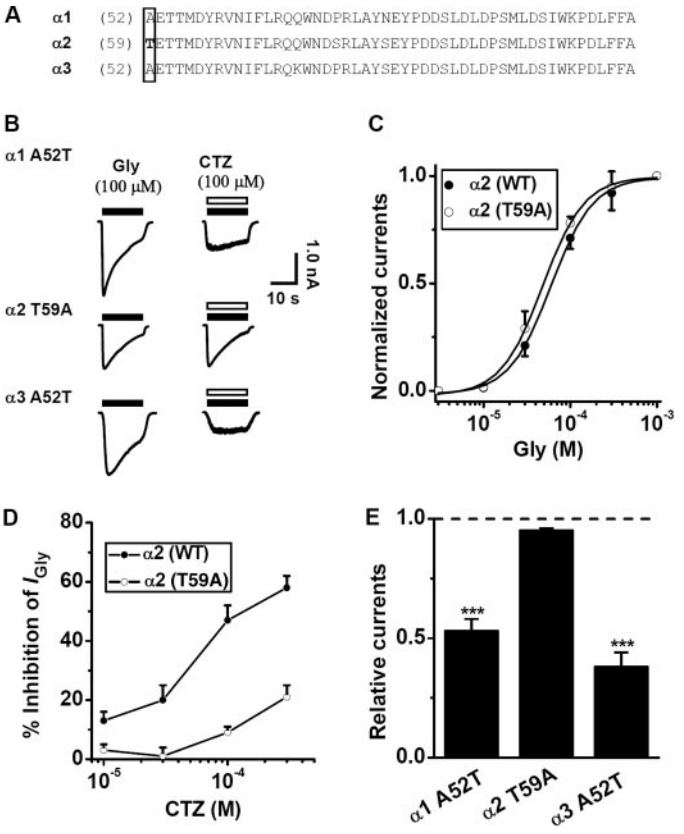

Fig. 5. The effects of CTZ on I_{Gly} mediated by mutant homomeric α1 (A52T), α2 (T59A), and α3 (A52T) GlyRs. A, part of amino acid sequence of α1, α2, and α3 subunits in the extracellular N terminus shows the difference of Thr59 in the α2 subunit with A52 in the α1 and α3 subunits. B, representative traces showing the effect of CTZ (100 μM) on I_{Gly} mediated by mutant homomeric α1 (A52T), α2 (T59A), and α3 (A52T) GlyRs expressed in HEK293T cells. C, concentration-response curves for I_{Gly} in HEK293T cells expressing WT α2 and mutant α2 (T59A) GlyR subunits (n = 6–12). D, percent inhibition of CTZ on I_{Gly} induced by EC_{70} glycine concentration [100 μM for WT α2-GlyR and 80 μM for mutant α2 (T59A)-GlyR] in HEK293T cells expressing WT α2 and mutant α2 (T59A) GlyR. (n = 6–12). E, histograms showing the relative I_{Gly} in the presence of CTZ (100 μM) in HEK293T cells transfected with α1 (A52T), α2 (T59A), or α3 (A52T) GlyR subunits (n = 6–12). ***, $P < 0.001$, compared with I_{Gly} before CTZ application (dashed line).

Therefore, these data indicate that Thr59 at the N terminus of α2 subunit is critical for the specific inhibition of α2-GlyRs by CTZ.

To establish that Thr59 residue specifically contributes to the CTZ inhibition of α2-GlyRs, we further performed reversal mutation in two insensitive subunits (α1 and α3) to see whether they can impart sensitivity to CTZ. Mutant homomeric α1 (A52T) and α3 (A52T) GlyRs, corresponding to position of Thr59 in α2 subunit, respectively (Fig. 5A), were examined. Note that CTZ (100 μM) significantly inhibited I_{Gly} induced by 100 μM glycine in HEK293T cells expressing mutated form of α1 (A52T) and α3 (A52T) GlyRs (Fig. 5E). Taken together, we conclude that Thr59 at the N terminus of α2 subunit confers the specific inhibition of α2-GlyRs by CTZ.

CTZ Inhibition Was Developmentally Regulated in Spinal Cord. A developmental switch from α2 homomers to $\alpha1\beta$ heteromers has been reported for spinal GlyRs (Becker et al., 1988). This differential expression pattern promotes us to examine whether CTZ inhibition of I_{Gly} declined with time in cultured spinal neurons. As shown in Fig. 6, CTZ markedly inhibited I_{Gly} in neurons 5 to 7 days in vitro (DIV), whereas in neurons of DIV 12 to 14 and 19 to 21, the inhibition became largely attenuated. Consistent with the predominant expression of the α2 subunit in the hippocampus throughout the developmental stage, no significant difference about CTZ inhibition was observed in cultured hippocampal neurons during development. This specific decline of CTZ inhibition on I_{Gly} in cultured spinal neurons further supports the notion that the CTZ action is α2 subunit-specific.

Discussion

The main finding of this study was that CTZ, an epilepigenic agent that interacts with both AMPA (Patneau et al., 1993) and $GABA_A$ receptors (Deng and Chen, 2003), specifically inhibited α2-GlyRs in both CNS neurons and HEK293T cells. The inhibition was glycine concentration-dependent, suggesting a competitive mechanism. Furthermore, a single mutation of T59A in the α2 subunit markedly reduced the inhibitory effect of CTZ. Because the α2-GlyR represents the major component of GlyRs in adult cortical neurons (Malosio et al., 1991), CTZ is useful to explore the role of GlyRs in the brain.

Several lines of evidence suggest it is very unlikely that CTZ acts as an open channel blocker. Unlike open channel blockers, brief exposure to CTZ before glycine application can result in inhibition of I_{Gly}. Furthermore, the CTZ effect on GlyR was independent of membrane voltage and was use-independent. In addition, the action of CTZ differs from that of the known open channel blocker PTX in that CTZ blocks homomeric and heteromeric α2-containing receptors, whereas PTX only blocks homomeric GlyRs (Yoon et al., 1993; Wang et al., 2006).

The mutation of A52S in α1 subunit was shown to decrease glycine sensitivity on GlyRs (Saul et al., 1994). In contrast, our results indicate the mutation of T59A, the corresponding site in the α2 subunit, only slightly altered the glycine sensitivity. However, T59A mutation largely eliminated the inhibitory effect of CTZ on α2-GlyR. Therefore, threonine in position 59 of the α2 subunit is critical for CTZ inhibition. Further support for Thr59 as the key residue conferring CTZ specificity comes from the results that CTZ became effective in inhibiting GlyRs containing α1 or α3 subunits when the site corresponding to Thr59 of the α2 subunit was changed to threonine (A52T). These data support the idea that a single amino acid at the extracelluar N terminus can dramatically affect the regulatory properties of GlyRs (Mascia et al., 1996).

Through site-directed mutagenesis combined with homology modeling based on the crystal structure of the acetylcholine binding protein, the ligand binding residues of α1 GlyR were investigated by previous studies (Lynch, 2004; Grudzinska et al., 2005). Ala52 of the α1 subunit is not one of the binding sites that were identified through homology modeling. Thus, Thr59 in the α2 subunit, the corresponding residue of Ala52 in the α1 subunit, may also not be the binding sites for glycine binding sites. However, Ala52 is one of the closest residues to the putative glycine binding sites as revealed by homology modeling (Speranskiy et al., 2007). In addition, the mutation of A52S in α1 subunit was shown to decrease glycine sensitivity of GlyRs (Saul et al., 1994).

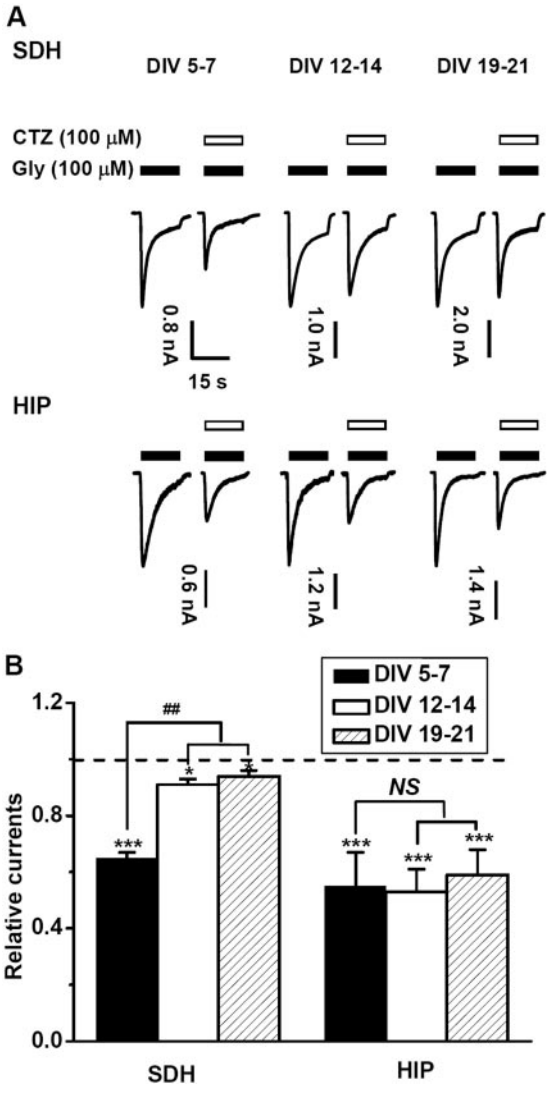

Fig. 6. Effects of CTZ on I_{Gly} in spinal cord and hippocampal neurons of various times in culture. A, typical traces showing I_{Gly} in the absence or presence of CTZ in DIV 5 to 7, 12 to 14, and 19 to 21 neurons from SDH and hippocampus (HIP), respectively. B, summary data showing the degree of CTZ inhibition of I_{Gly} with time in culture. *, $P < 0.05$; ***, $P < 0.001$ compared with I_{Gly} before CTZ application (dashed line). ###, $P < 0.001$; *NS*, no significant difference. n = 7–10.

These studies suggest that Ala52 in α1 GlyR is relevant to the glycine binding site. Accordingly, Thr59 in the α2 subunit, the corresponding residue of Ala52 in the α1 subunit, may play a similar role in glycine binding through which CTZ exerts the competitive inhibition of GlyRs.

Previous studies have indicated that the expression of GlyR subunits in rat spinal cord neurons in vivo was developmentally regulated (Malosio et al., 1991; Watanabe and Akagi, 1995). The α1 and β subunits are expressed at very low levels in embryonic rat spinal cord, with increasing expression during the first 2 postnatal weeks and the sustained high level thereafter. The α3 subunit is expressed after the 3rd postnatal week and only at a low level. In contrast, α2 expression is high and widespread in the embryonic nervous system and decreases gradually after birth. Similar developmental regulation of GlyR subunits expression has also been observed in primary cultures of spinal cord neurons; mRNA for α1 increased by 1.5- to 2-fold, whereas that for α2 decreased substantially over the first 10 days in culture (Bechade et al., 1996). Consistent with the selective inhibition of CTZ on α2-GlyRs expressed in HEK293T cells, we found that CTZ significantly inhibited I_{Gly} in DIV 5 to 7 spinal dorsal horn neurons, but the inhibition was greatly reduced after DIV 12. These results show that CTZ inhibition of GlyRs in spinal cord is developmentally regulated, reflecting the α2-to-α1 switch of GlyR subunit (Bechade et al., 1996; Aguayo et al., 2004) and support the notion of α2 subunit specificity of the CTZ action.

Unlike the high level of GlyR α1 subunit expression in the mature spinal cord, the α2- rather than α1-containing GlyR is the predominant form in the hippocampus (Malosio et al., 1991; Thio et al., 2003). The physiological consequences of this distinct distribution pattern are difficult to establish because of the lack of specific antagonists. Thus, CTZ may be a useful tool to study the molecular organization and function of GlyRs in the hippocampus. Furthermore, recent studies have shown that the activation of α2-GlyRs is excitatory in developing cortex (Sturman, 1993; Flint et al., 1998) and plays an important role in rod photoreceptor development (Young and Cepko, 2004) and interneuron differentiation (McDearmid et al., 2006). However, genetic deletion of the α2 subunit in mice has not yielded clear developmental deficiency (Young-Pearse et al., 2006). This may be accounted for the compensatory expression of other α subunits in these α2 knockout mice (Kling et al., 1997). Therefore, the acute pharmacological inhibition of α2-GlyRs by CTZ may be helpful for studying the role of α2-GlyRs in neuronal development.

The endogenous amino acids, glycine, taurine and β-alanine, mediate tonic activation of α2-GlyRs, which maintains inhibitory tone in the hippocampus (Mori et al., 2002). Moreover, the synaptic transmission in the rat hippocampus was also shown to be depressed by activation of GlyRs (Chattipakorn and McMahon, 2003; Song et al., 2006). These findings indicate that GlyRs may play an important role in mediating inhibitory neurotransmission in the hippocampus. Recent studies have provided evidence that, in addition to enhancing the function of glutamate receptors, CTZ affects the output of neural networks by reducing GABAergic inhibition (Deng and Chen, 2003; Qi et al., 2006). Our present study showed that CTZ inhibited both glycine- and taurine-evoked currents in cultured rat hippocampal neurons. These results thus suggest that CTZ may inhibit the tonic activation of α2-GlyRs by taurine and glycine and further accelerate the overall neuronal activity within the hippocampus, an aspect not considered in previous studies on the CTZ action in enhancing hippocampal excitability. Given its enhancing effect on AMPA receptors and inhibitory effect on glycine or $GABA_A$ receptors, CTZ might be a superior epileptogenic agent. Note that CTZ-induced epileptiform activity in cultured hippocampal neurons is not a transient change but rather a permanent alteration of neural networks (Qi et al., 2006). Because α2-GlyRs may promote interneuron development (McDearmid et al., 2006), it is possible that the long-lasting effect of CTZ on overall network output in developing neural network may result from the impairment of interneuron differentiation as a result of its inhibition of α2-GlyRs. In addition, previous studies also indicated that the GlyR α2 subunit mRNA as well as functional GlyRs are expressed in other forebrain areas, such as hypothalamus, dorsal striatum, and amygdala (McCool and Farroni, 2001; Sergeeva and Haas, 2001). Therefore, CTZ may also modulate neuronal excitability through inhibiting GlyR in these forebrain regions.

Both inhibitory glycine and $GABA_A$ receptors belong to the cysteine loop superfamily of ligand-gated ion channels. The homology between these two receptors may account for the similar inhibitory effect of CTZ. Unraveling the molecular mechanisms underlying the CTZ action on both glycine and $GABA_A$ receptors may yield critical insight into the specificity and mechanism of drug-receptor interactions.

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Correction to "α2 Subunit Specificity of Cyclothiazide Inhibition on Glycine Receptors"

In the above article [Zhang XB, Sun GC, Liu LY, Yu F, and Xu TL (2008) *Mol Pharmacol* **73:**1195–1202], the corresponding author's e-mail address is incorrect. The correct address is tlxu@ion.ac.cn.

The online version will be corrected in departure from the print version.

The printer regrets this error and apologizes for any confusion or inconvenience it may have caused.