# Cyclothiazide potently inhibits $\gamma$-aminobutyric acid type A receptors in addition to enhancing glutamate responses

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**Ionotropic glutamate and $\gamma$-aminobutyric acid type A ($GABA_A$) receptors mediate critical excitatory and inhibitory actions in the brain. Cyclothiazide (CTZ) is well known for its effect of enhancing glutamatergic transmission and is widely used as a blocker for $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor desensitization. Here, we report that in addition to its action on AMPA receptors, CTZ also exerts a powerful but opposite effect on $GABA_A$ receptors. We found that CTZ reversibly inhibited both evoked and spontaneous inhibitory postsynaptic currents, as well as GABA application-induced membrane currents, in a dose-dependent manner. Single-channel analyses revealed further that CTZ greatly reduced the open probability of $GABA_A$ receptor channels. These results demonstrate that CTZ interacts with both glutamate and $GABA_A$ receptors and shifts the excitation–inhibition balance in the brain by two independent mechanisms. Understanding the molecular mechanism of this double-faceted drug–receptor interaction may help in designing new therapies for neurological diseases.**

Cyclothiazide (CTZ) was developed originally as a diuretic drug with clinical application in treating hypertension (1). In the early 1990s, CTZ was found to block the desensitization of $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors and prolong glutamatergic synaptic currents (2, 3). Among a large family of thiazides, CTZ has been proven to be the most potent and therefore the most extensively studied and widely used positive AMPA receptor modulator (4–10). In addition to a postsynaptic allosteric modulation of AMPA receptors, CTZ has also been found to exert a presynaptic effect by increasing the frequency of spontaneous glutamate release (5, 7, 8).

Because AMPA receptors are important in mediating excitatory neurotransmission in the CNS, drugs positively modulating AMPA receptors have been explored for possible therapeutic applications (11). On the positive side, AMPA receptor modulators enhancing glutamate neurotransmission were found to facilitate long-term potentiation and improve learning and memory (11–14). On the negative side, administration of CTZ might cause seizures and cell death (15–17). So far, all of the effects induced by CTZ have been interpreted in the context of antagonistic action on AMPA receptor desensitization. Here we show that in addition to enhancing glutamatergic transmission, CTZ also strongly inhibits $\gamma$-aminobutyric acid (GABA) type A ($GABA_A$) receptors and diminishes GABAergic transmission.

## Methods

**Cell Culture.** Hippocampal microisland cultures were prepared from newborn Sprague–Dawley rats as described (18, 19). In brief, the hippocampal CA1–CA3 region was dissected and incubated for 30 min in 0.05% trypsin/EDTA solution (pH 7.2). After enzyme treatment, tissue blocks were triturated gently by using a fire-polished Pasteur pipette. Dissociated cells were plated onto microislands (0.4 mM poly-D-lysine/0.25 mM collagen) covered by a monolayer of astrocytes. The culture medium contained 500 ml of MEM (GIBCO), 5% FBS (HyClone), 10 ml of B-27 supplement (GIBCO), 100 mg of $NaHCO_3$, 20 mM D-glucose, 0.5 mM L-glutamine, and 25 units/ml penicillin/streptomycin. Cells were maintained in a 5% $CO_2$/95% air incubator for up to 3–4 weeks.

**Electrophysiology.** Whole-cell and outside-out patch recordings were performed by using Multiclamp 700A and Axopatch 200B patch-clamp amplifiers (Axon Instruments, Foster City, CA). Patch pipettes were pulled from borosilicate glass and fire-polished. The final resistance of pipettes was 2–4 M$\Omega$ for whole-cell and 6–10 M$\Omega$ for single-channel recordings. The recording chamber was perfused continuously with a bath solution that consisted of 128 mM NaCl, 30 mM D-glucose, 25 mM Hepes, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ (pH 7.3, adjusted with NaOH). The pipette solution for whole-cell recordings contained 147 mM CsCl, 5 mM disodium phosphocreatine, 2 mM EGTA, 10 mM Hepes, 2 mM MgATP, and 0.3 mM $Na_2GTP$ (pH 7.3, adjusted with CsOH). For evoked inhibitory postsynaptic currents (IPSCs), 120 mM CsCl was replaced with equivocal $CsMeSO_3$ to reduce the IPSC amplitude for better voltage control. For single-channel recordings, pipettes were coated with wax and filled with a solution that contained 120 mM CsCl, 20 mM tetraethylammonium-Cl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, and 10 mM Hepes (pH 7.3, adjusted with CsOH). In whole-cell recordings, the series resistance was typically 10–20 M$\Omega$ and compensated by 50–70%. Data were acquired by using PCLAMP 8 software, sampled at 10 kHz and filtered at 1–2 kHz, and analyzed with CLAMPFIT 8 or CLAMPFIT 9 software (Axon Instruments). Miniature events were analyzed by using MINIANALYSIS software (Synaptosoft, Decatur, GA). Analyzed data were expressed as mean $\pm$ SE, and a paired Student $t$ test was used for most comparisons between control and CTZ-treated groups, unless stated otherwise.

**Drugs.** CTZ was purchased from Sigma and Tocris Cookson (St. Louis) and dissolved in either DMSO or ethanol. The inhibitory effect of CTZ on $GABA_A$ receptors was similar irrespective of the source or solvent. The vehicle control for DMSO and ethanol had no significant effect on GABA-mediated responses at low concentrations ($\leq$0.1%). When a high concentration of CTZ (500–1,000 $\mu$M) was used, DMSO was used to dissolve CTZ, and an equivalent concentration of DMSO was included in the control solution to diminish the solvent effect. Bicuculline (BIC), 6-nitro-7-cyanoquinoxaline-2,3-dione, tetrodotoxin, and GABA were obtained from Tocris Cookson. All of the drugs were

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Abbreviations: AMPA, $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BIC, bicuculline; CTZ, cyclothiazide; GABA, $\gamma$-aminobutyric acid; $GABA_A$, GABA type A; EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; mEPSC/IPSC, miniature EPSC/IPSC; $NP_o$, open probability.

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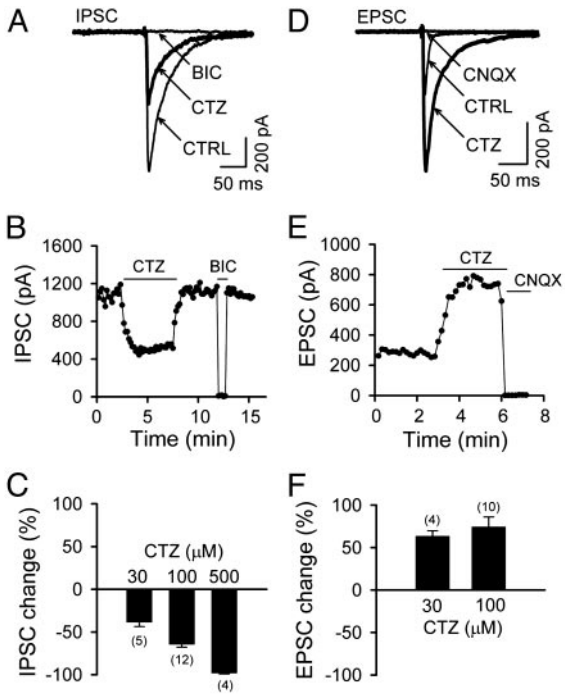


**Fig. 1.** CTZ potently inhibits IPSCs but enhances EPSCs. (*A*) A typical example showing evoked autaptic IPSCs (average five to eight traces) in control (CTRL), 100 $\mu$M CTZ, and specific $GABA_A$ receptor antagonist BIC (40 $\mu$M). Holding potential was −70 mV. (*B*) Time-effect plot illustrating the amplitude of IPSCs reversibly blocked by CTZ and BIC. (*C*) Pooled data showing that CTZ inhibited IPSCs in a dose-dependent manner. (*D* and *E*) CTZ (100 $\mu$M) enhanced the amplitude and prolonged the decay of evoked autaptic EPSCs in a representative neuron. CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione. (*F*) Pooled data showing the percentage of EPSC amplitude increase by CTZ.

freshly diluted in bath solution to final concentrations before experiments.

## Results

**Opposite Effects of CTZ on Evoked IPSCs and Excitatory Postsynaptic Currents (EPSCs).** Evoked synaptic currents were recorded from single autaptic neurons by a brief depolarization (−70 mV to 0 mV, 1.5 ms) in hippocampal microisland cultures (18, 19). CTZ is well known as an antagonist of AMPA receptor desensitization. During our studies of synaptic transmission, CTZ was applied when evoked IPSCs, instead of EPSCs, were recorded from autaptic neurons. Surprisingly, 100 $\mu$M CTZ, a concentration commonly used for the study of EPSCs (5, 8), potently reduced the amplitude of IPSCs (Fig. 1*A*). The IPSCs were blocked by BIC (40 $\mu$M), demonstrating their mediation by $GABA_A$ receptors. The CTZ effect on IPSCs was reversible (Fig. 1*B*) and dose-dependent (Fig. 1*C*). As summarized in Fig. 1*C*, IPSCs were almost abolished by a high concentration of CTZ (500 $\mu$M, 98.4 ± 0.8% reduction, $n = 4$) and significantly inhibited at low to medium concentrations (30 $\mu$M, 38.7 ± 4.9% reduction, $n = 5$; 100 $\mu$M, 64.6 ± 3.3% reduction, $n = 12$). As reported (5, 9), the same concentration of CTZ enhanced evoked autaptic EPSCs recorded from single glutamatergic neurons (Fig. 1 *D* and *E*). Potentiation of EPSCs by CTZ was seen at similar concentrations (30 $\mu$M, 163.7 ± 6.1% of control, $n = 4$; 100 $\mu$M, 174.5 ± 11.6% of control, $n = 10$) as the inhibition of IPSCs. These results demonstrate a dual effect of CTZ in modulating two different synaptic transmission systems: to enhance glutamatergic transmission and to inhibit GABAergic transmission, both of which will lead to an increase of neuronal activity.

**Effect of CTZ on Spontaneous Release Events.** We next examined whether the CTZ-mediated inhibition of GABAergic transmission was due to changes in pre- or postsynaptic activity. To discriminate between pre- and postsynaptic action, we tested the effect of CTZ on miniature IPSCs (mIPSCs) in the presence of tetrodotoxin (1 $\mu$M) and 6-nitro-7-cyanoquinoxaline-2,3-dione (10 $\mu$M). In general, a significant change in the frequency of spontaneous releasing events would likely indicate a presynaptic effect, whereas a significant change in amplitude would indicate a postsynaptic effect. Fig. 2*A1* shows representative traces of mIPSCs in control that were blocked by BIC and were, thus, GABAergic (BIC data not shown). In the presence of CTZ (100 $\mu$M), both the amplitude and frequency of mIPSCs were greatly reduced (Fig. 2*A2*). The cumulative fraction plot in Fig. 2*A3* shows a left shift of the amplitude of mIPSCs toward smaller values in the presence of CTZ. Data obtained from 12 neurons are summarized in Fig. 2 *C* and *D* to show a significant decrease in both the amplitude (control, 35.2 ± 1.2; CTZ, 24.6 ± 1.7; $P < 0.001$) and frequency (control, 1.22 ± 0.21; CTZ, 0.54 ± 0.15; $P < 0.001$) of mIPSCs in the presence of CTZ. The marked reduction in the amplitude of BIC-sensitive mIPSCs suggests that CTZ may exert a postsynaptic effect on $GABA_A$ receptors. The CTZ inhibition of the mIPSC amplitude was probably an underestimate because many originally small mIPSCs might have been too small to be detected after the CTZ inhibition, which made the mIPSC amplitude during CTZ application larger than the true value. The frequency reduction of mIPSCs in the presence of CTZ may point to an additional presynaptic effect but also may be caused indirectly by the substantial reduction of the amplitude.

In contrast to the effect on mIPSCs, CTZ significantly enhanced both the frequency and amplitude of mEPSCs, as reported (5, 9). Typical traces of control mEPSCs recorded in the presence of tetrodotoxin (1 $\mu$M) and BIC (40 $\mu$M) are shown in Fig. 2*B1*. Application of CTZ (100 $\mu$M) increased the frequency and right-shifted the amplitude of mEPSCs toward larger values (Fig. 2 *B2* and *B3*). In Fig. 2 *E* and *F*, data pooled from 11 neurons showed a consistent increase in the amplitude (control, 29.1 ± 3.0; CTZ, 40.4 ± 4.3; $P < 0.001$) and frequency of mEPSCs in the presence of CTZ (control, 1.00 ± 0.17; CTZ, 2.34 ± 0.37; $P < 0.001$).

**CTZ Inhibition of GABA-Application-Induced Membrane Currents.** The marked reduction of mIPSC amplitude in the presence of CTZ prompted us to examine further its postsynaptic effect on $GABA_A$ receptors. Direct bath application of GABA (40 $\mu$M) under a whole-cell voltage clamp condition (holding potential −70 mV) induced a rapidly decaying inward current (Fig. 3*A*). In the presence of CTZ (100 $\mu$M for 60 s), the same concentration of GABA induced a much smaller current (Fig. 3*B*), suggesting that CTZ may act as an antagonist for $GABA_A$ receptors. Bath application of CTZ (100 $\mu$M) in the absence of GABA did not evoke any membrane currents (Fig. 3*C*). To test whether CTZ acts rapidly in blocking $GABA_A$ receptors, drug application was rapidly switched between GABA and GABA plus CTZ. As shown in Fig. 3*D*, the blocking effect of CTZ on $GABA_A$ receptors occurred instantaneously, implying a direct interaction rather than mediation through a cell-signaling pathway. The $IC_{50}$ calculated from the dose–response curve of CTZ inhibition on the peak amplitude of GABA-evoked currents was 57.6 $\mu$M (Fig. 3*E*). This value appears to be similar to the $IC_{50}$ for CTZ inhibition of evoked IPSCs (compare Figs. 3*E* and 1*C*), confirming a major postsynaptic effect of CTZ on GABAergic transmission. To examine whether the CTZ inhibition was voltage dependent, membrane potentials were changed from −70 mV to 50 mV when recording the GABA-evoked currents in the presence of CTZ (100 $\mu$M). No significant difference in the percentage inhibition was found across the 120-mV range

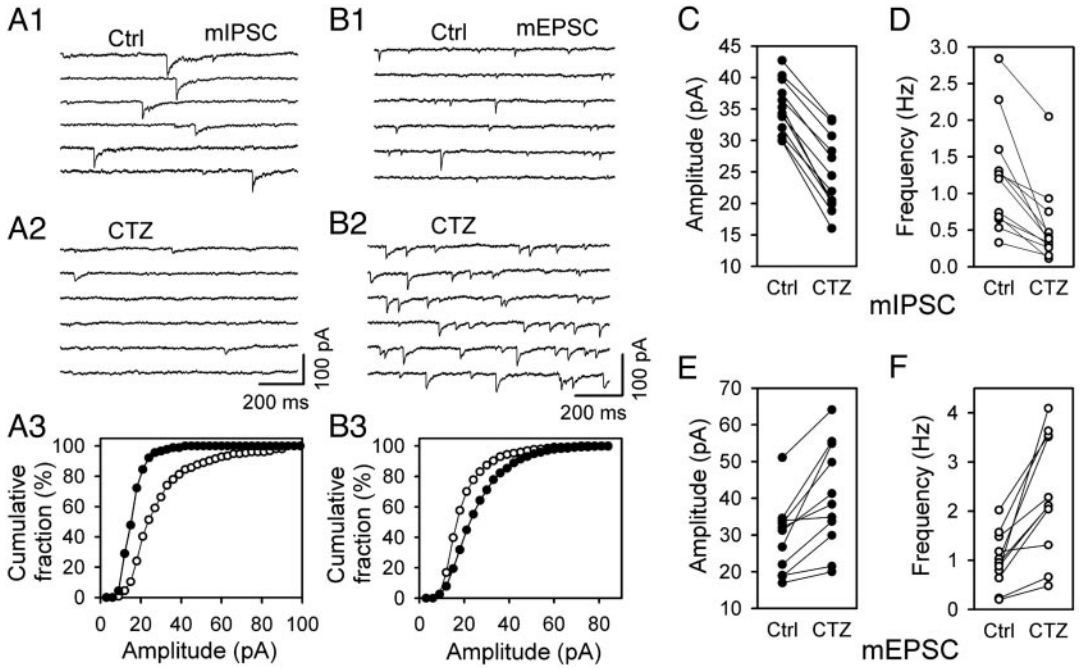


**Fig. 2.** CTZ decreases spontaneous mIPSCs but increases mEPSCs. (*A1*) Consecutive traces showing control (Ctrl) mIPSCs. (*A2*) CTZ (100 $\mu$M) greatly inhibited mIPSCs. (*A3*) Cumulative fraction plot illustrating the decrease of mIPSC amplitude in the presence of CTZ ($P < 0.001$, Kolmogorov–Smirnov test; ○, Ctrl; ●, CTZ). (*B1*) Typical example showing Ctrl mEPSCs. (*B2*) CTZ (100 $\mu$M) significantly enhanced mEPSCs. (*B3*) Cumulative fraction plot demonstrating the increase of mEPSC amplitude in the presence of CTZ ($P < 0.001$; ○, Ctrl; ●, CTZ). (*C*) Pooled data showing consistent reduction of mIPSC amplitude by CTZ. (*D*) Pooled data showing marked reduction of mIPSC frequency by CTZ. (*E*) Pooled data illustrating significant enhancement of mEPSC amplitude by CTZ. (*F*) Pooled data illustrating remarkable increase of mEPSC frequency by CTZ. $P < 0.001$, paired *t* test for *C–F*.

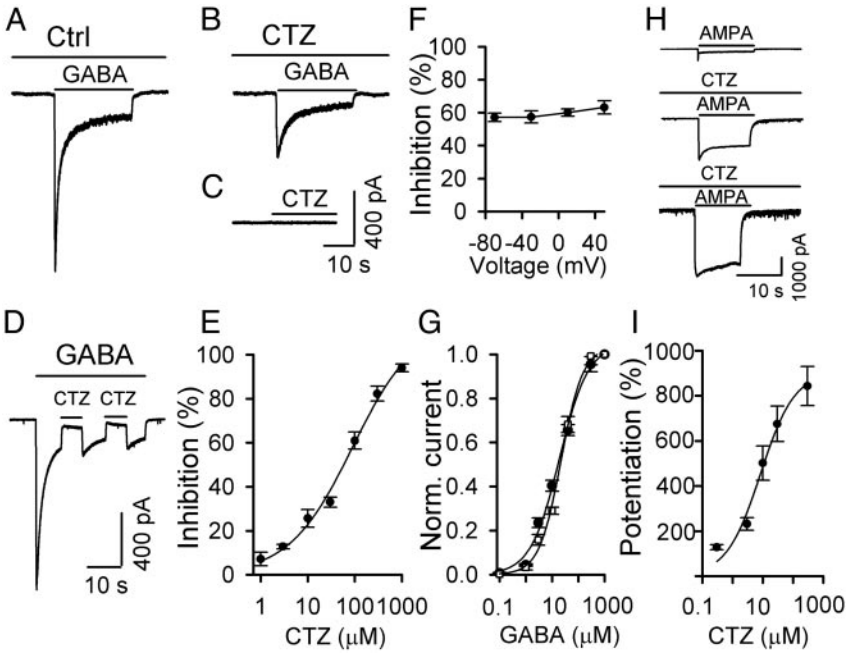


**Fig. 3.** CTZ blocks GABA-induced postsynaptic receptor responses. (*A*) Control (Ctrl) trace showing an inward current induced by bath application of GABA (40 $\mu$M). (*B*) CTZ (100 $\mu$M) inhibited the GABA-induced membrane current significantly. (*C*) Application of CTZ (100 $\mu$M) alone did not induce any membrane currents. (*D*) CTZ blocked the GABA-induced current instantaneously during rapid switch between GABA and GABA plus CTZ application. (*E*) Dose–response curve of CTZ inhibition on the peak amplitude of GABA-evoked inward currents. $IC_{50} = 57.6$ $\mu$M. (*F*) CTZ (100 $\mu$M) inhibition of GABA-evoked currents at different holding potentials. No significant difference was detected ($P > 0.36$, one-way ANOVA). (*G*) GABA dose–response curve in the absence (●) and presence (□) of CTZ (100 $\mu$M). $EC_{50GABA} = 18.8$ $\mu$M. $EC_{50GABA+CTZ} = 22.2$ $\mu$M. (*H*) Representative traces showing inward currents induced by bath application of AMPA (10 $\mu$M, *Top*), AMPA plus 10 $\mu$M CTZ (*Middle*), and AMPA plus 300 $\mu$M CTZ (*Bottom*). (*I*) Dose–response curve of CTZ potentiation of AMPA-evoked peak currents. $EC_{50} = 10.4$ $\mu$M. The number of experiments in *E–G* and *I* ranged from four to nine, with the majority being six or seven.

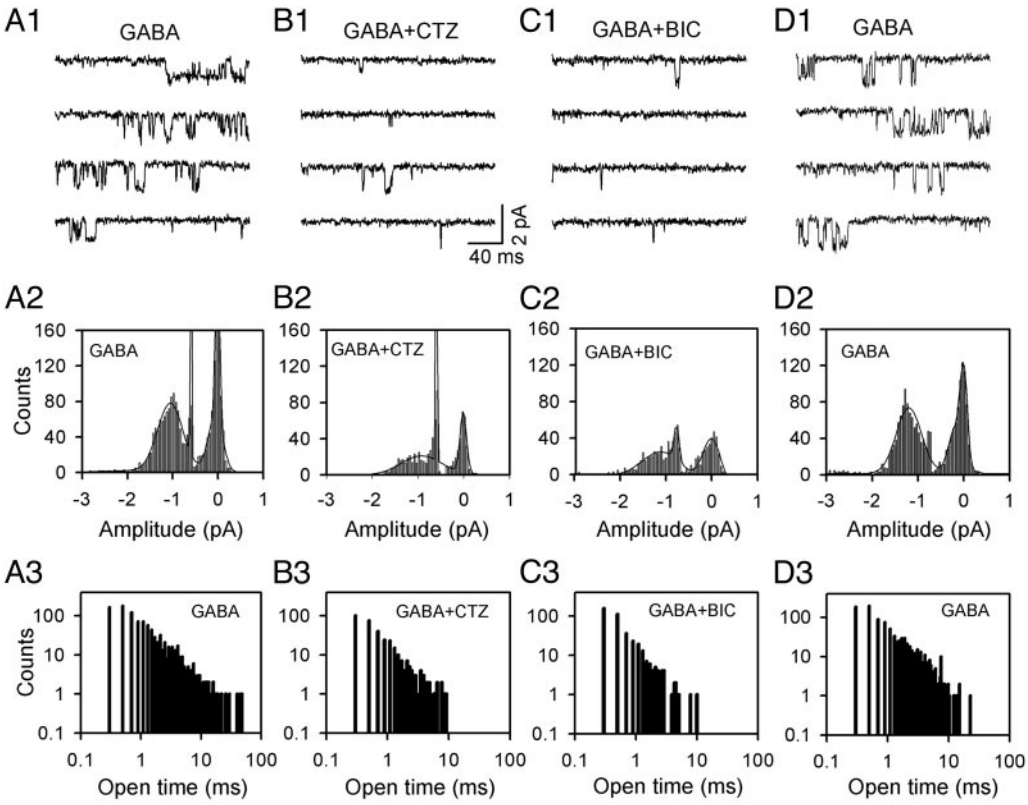


**Fig. 4.** CTZ inhibits $GABA_A$ receptor single-channel activity. (*A1*) Representative traces showing single-channel currents activated by bath perfusion of GABA (2 $\mu$M) from an outside-out patch. Channel openings are downward. (*A2*) The amplitude-distribution histogram of GABA-activated single-channel currents. (*A3*) The open time-distribution histogram of GABA-activated single-channel currents. (*B1–B3*) Representative traces (*B1*), amplitude histogram (*B2*), and open time-distribution histogram (*B3*) of single-channel currents in the presence of GABA plus CTZ (100 $\mu$M). Note a great reduction in the number of open events and a disappearance of long openings (>10 ms) in the presence of CTZ. (*C1–C3*) Representative traces (*C1*), amplitude histogram (*C2*), and open time-distribution histogram (*C3*) of single-channel currents in the presence of GABA plus BIC (40 $\mu$M). (*D1–D3*) Recovery of GABA-evoked single-channel events after washing off the drugs. All results in *A–D* were obtained from the same patch with the holding potential at −70 mV.

(Fig. 3*F*; $P > 0.36$, one-way ANOVA). We further tested whether CTZ interferes with GABA binding by analyzing the effect of CTZ on the GABA dose–response curve. The $EC_{50GABA}$ value was 18.8 $\mu$M, similar to previous reports (20, 21). In the presence of CTZ (100 $\mu$M), the GABA dose–response curve was not apparently shifted ($EC_{50GABA+CTZ}$ = 22.2 $\mu$M), suggesting that CTZ may have only a slight effect, if any, on GABA binding (Fig. 3*G*).

In contrast to the inhibitory effect of CTZ on GABA-evoked currents, bath application of CTZ greatly potentiated AMPA-evoked currents (10 $\mu$M; Fig. 3*H*). The potentiation of CTZ on AMPA receptor currents was also dose dependent, with an $EC_{50}$ value of 10.4 $\mu$M (Fig. 3*I*), similar to previous studies (2, 22).

**Single-Channel Analysis of CTZ Action on $GABA_A$ Receptors.** The inhibitory effect of CTZ on bath application of GABA-induced membrane currents suggests a possible direct interaction between CTZ and $GABA_A$ receptors. To test this possibility, we performed single-channel recordings to examine whether CTZ can inhibit GABA-activated single-channel currents. Outside-out patches were excised from neuronal soma to monitor single-channel events in response to bath perfusion of GABA (2 $\mu$M), GABA plus CTZ (100 $\mu$M), GABA plus BIC (40 $\mu$M), and GABA after washing off the drugs (Fig. 4). Bath application of GABA activated many single-channel openings, with some of the duration lasting longer than 10 ms (Fig. 4 *A1* and *A3*). As expected, the $GABA_A$ receptor channels displayed multiple conductance states (Fig. 4 *A1* and *A2*). The main conductance of the single-channel currents was ≈27 pS (Fig. 5 *C* and *D*), consistent with previous reports (23, 24). When CTZ was applied with GABA, the number of single-channel events was greatly reduced (Fig. 4 *B1* and *B2*). The channel open duration in the presence of CTZ was rarely longer than 10 ms (Fig. 4*B3*). To confirm that the single-channel events were indeed GABA activated, we applied BIC together with GABA on the same patch and found that the single-channel events were also greatly inhibited (Fig. 4*C*). At the single-channel level, the CTZ inhibition was also reversible, demonstrated by the recovery of GABA-activated single-channel events after washing off drugs (Fig. 4*D*). In a total of 11 outside-out patches, we found a consistent inhibition by CTZ and BIC on the single-channel open frequency and open probability ($NP_o$; Fig. 5 *A* and *B*). Although CTZ greatly inhibited the number of channel events, the main conductance of $GABA_A$ receptor channels was only slightly reduced by CTZ (≈8% decrease; Fig. 5 *C* and *D*). These results at the single-channel level suggest that CTZ may bind directly to $GABA_A$ receptors and block channel openings without a great effect on the channel conductance.

## Discussion

The inhibitory effect of CTZ on $GABA_A$ receptors, in addition to its well known effect of enhancing glutamate responses, places CTZ at a unique position in modulating neuronal activities in the brain. Because glutamate and GABA are the most abundant excitatory and inhibitory neurotransmitters in the CNS, respectively, introduction of CTZ into a neural network may greatly

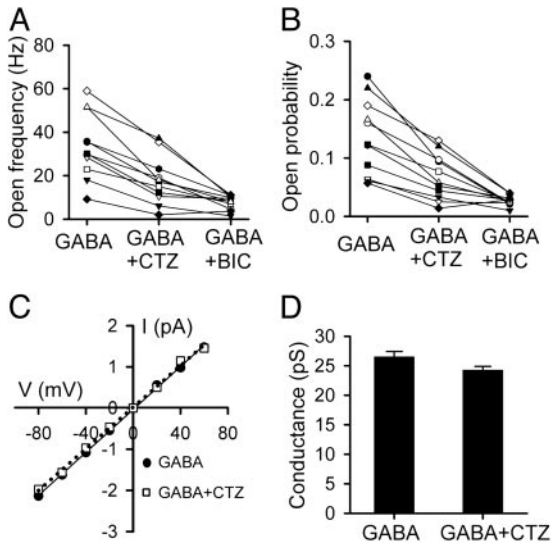


**Fig. 5.** CTZ reduced the $GABA_A$ receptor channel $NP_o$ without marked effect on the channel conductance. (*A*) The channel open frequency was consistently reduced by CTZ (100 $\mu$M) and BIC (40 $\mu$M). (*B*) The channel $NP_o$ was also similarly reduced by CTZ and BIC ($P < 0.001$, for both CTZ and BIC in *A* and *B*). (*C*) Current–voltage (*I–V*) curve showing similar main $GABA_A$ receptor channel conductance ($\approx$27 pS) with or without CTZ in the bath solution. (*D*) Bar graph illustrating only a slight reduction of the channel conductance by CTZ.

shift the excitation–inhibition balance and dramatically accelerate the overall neuronal activity within a neural circuit. This possibility may raise considerable interest in making use of this special effect of CTZ in certain circumstances. For example, when an increase of neuronal activity is necessary, CTZ may be superior to other drugs that act only on either glutamate or GABA receptors alone. On the other hand, overdose of CTZ may cause potential excitotoxicity because of its one-way drive toward excitation.

The potent but opposite effects of CTZ on glutamate and GABA receptors may also have important implications in studying molecular mechanisms of drug–receptor interactions. The molecular structures of AMPA receptors and $GABA_A$ receptors are distinct, yet apparently they both possess a binding site for CTZ. More interestingly, the binding of CTZ leads to increased $NP_o$ of AMPA receptor channels but decreased $NP_o$ of $GABA_A$ receptor channels. This effect is reminiscent of the opposing effects of barbiturates. However, barbiturates enhance $GABA_A$ receptors and inhibit glutamate receptors (25, 26). CTZ does not resemble barbiturates in terms of chemical structure, but the possibility that they might act at the same site yet have opposite effects on both GABA and glutamate receptors cannot be ruled out. Unraveling the molecular mechanisms of the opposite interactions between CTZ and glutamate and $GABA_A$ receptors may yield critical insight for the development of new drugs to combat receptor dysfunction-related diseases.

In light of the newly discovered inhibitory effect of CTZ on $GABA_A$ receptors, some previous CTZ studies may have overstated the importance of glutamate receptor desensitization in determining the output of neural networks. Positive modulators of glutamate receptors have been suggested to facilitate the formation of long-term potentiation and possibly enhance memory storage (11, 14). Our results suggest that the inhibition of GABAergic neurotransmission by CTZ may have contributed to the overall increase of neuronal activity and might also play a role in long-term potentiation and memory enhancement. In fact, previous studies have shown a direct involvement of the GABAergic transmission system in modulating long-term synaptic plasticity, including learning and memory (27–30). Better understanding of the coordination between glutamatergic and GABAergic transmission systems, as well as the development of new drugs that interact with both glutamate and GABA receptors, may be a rationale in the design of new memory enhancement therapies.

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