Zinc Enhances the Inhibitory Effects of Strychnine-Sensitive Glycine Receptors in Mouse Hippocampal Neurons

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In the hippocampus, tonic glycine application decreases neuronal excitability. To test this possibility, we examined paradigmatic effects of sustained glycine applications alone did not alter the number of action potentials evoked by depolarizing steps, but they did in 1 μM Zn2+. At least part of this effect resulted from Zn2+ enhancing the GlyR-induced decrease in input resistance. Sustained 20 μM glycine application alone did not alter neuronal bursting, a form of hyperexcitability induced by omitting extracellular Mg2+. However, sustained 20 μM glycine applications depressed neuronal bursting in 1 μM Zn2+, Zn2+ did not enhance the inhibitory effects of sustained 60 μM glycine in these paradigms. These results suggest that tonic GlyR activation could decrease neuronal excitability. To test this possibility, we examined the effect of the GlyR antagonist strychnine and the Zn2+ chelator tricine on action potential firing by CA1 pyramidal neurons in mouse hippocampal slices. Co-applying strychnine and tricine slightly but significantly increased the number of action potentials fired during a depolarizing current step and decreased the rheobase for action potential firing. Thus Zn2+ may modulate neuronal excitability normally and in pathological conditions such as seizures by potentiating GlyRs tonically activated by low agonist concentrations.

INTRODUCTION

Strychnine-sensitive glycine receptor (GlyR) expression occurs throughout the mammalian CNS including the forebrain (Lynch 2004). Brain stem and spinal cord GlyRs contribute to fast inhibitory synaptic transmission (Lynch 2004), but their role in the forebrain is less clear. In the hippocampus, GlyRs may contribute to tonic inhibition, because inhibitors of glycine, taurine, and β-alanine transport reveal a tonic GlyR-mediated current (Mori et al. 2002; Zhang et al. 2007). Recent studies have indicated that sustained GlyR activation reduces hippocampal neuronal excitability under normal (Song et al. 2006; Zhang et al. 2006) and hyperexcitable conditions (Chattpakorn and McMahon 2003; Kirchner et al. 2003).

Although hippocampal GlyRs have a different subunit composition from those in the brain stem and spinal cord (Chattipakorn and McMahon 2002; Mangin et al. 2005; Thio et al. 2003), extracellular Zn2+ is a potent and effective biphasic, endogenous modulator of GlyRs in all three regions (Bloomenthal et al. 1994; Chattipakorn and McMahon 2002; Laube et al. 1995; Miller et al. 2005; Thio and Zhang 2006; Ye et al. 2001). Zn2+ potentiates GlyRs at concentrations <10 μM and inhibits GlyRs at higher concentrations when exogenous agonist applications activate GlyRs in these areas. When synaptically released glycine activates GlyRs in the zebrafish brain stem (Suwa et al. 2001) and rodent spinal cord (Hirzel et al. 2006; Laube 2002), Zn2+ modulates the receptors in a similar manner. In addition, a hyperekplexia phenotype occurs in mice expressing mutant spinal GlyRs that extracellular Zn2+ cannot potentiate (Hirzel et al. 2006). However, the physiological significance of Zn2+ potentiation and inhibition of hippocampal GlyRs is less clear. We at least expect Zn2+ potentiation to be relevant because basal extracellular Zn2+ concentrations in the hippocampus are 2–3 μM (Kay 2003; Li et al. 2001). This potentiation may also contribute to the anticonvulsant effect of Zn2+ in some experimental models (Cole et al. 2000; Dominguez et al. 2003; Takeda et al. 2003). Here we examined the hypothesis that potentiating Zn2+ concentrations enhance the inhibitory effects of GlyRs under normal and hyperexcitable conditions. Some of these results have been presented previously in abstracts (Zhang and Thio 2006a,b).

METHODS

Preparations

The Washington University Animal Studies Committee approved all experimental protocols.

EMBRYONIC MOUSE HIPPOCAMPAL CULTURES. Hippocampal neurons were obtained from Swiss Webster mouse embryos at day 16 of gestation and cultured for 7–16 days as described previously (Thio et al. 2003). Dams were anesthetized with 5% halothane so that a paw pinch produced no response and killed by cervical dislocation. Embryos were obtained by cesarean section and killed by decapitation.

ACUTE MOUSE HIPPOCAMPAL SLICES. Acute hippocampal slices were prepared from 3-wk-old Swiss-Webster mice. Mice were decapitated after being anesthetized with 5% halothane. Brains were removed and immediately placed in modified ice cold sucrose containing physiological saline (Geiger and Jonas 2000). It was saturated with 95% O2–5% CO2 and contained (in mM) 87 NaCl, 25 NaHCO3, 2.5 KCl, 0.5 CaCl2, 7 MgCl2, 1.25 NaH2PO4, 75 sucrose, and 25 D-glucose. While in ice cold sucrose containing physiological saline, 350-μm-thick coronal brain slices were cut using a Vibratome Series.
Electrophysiology

WHOLE CELL PATCH-CLAMP ELECTROPHYSIOLOGY. Whole cell voltage- and current-clamp recordings were obtained from cultured neurons using an Axopatch 200A amplifier (Axon Instruments, Union City, CA) as described previously (Thio et al. 2003). CA1 pyramidal neurons for electrophysiological recordings were identified in acute hippocampal slices using infrared differential interference contrast (IR-DIC) video microscopy according to published methods (Stuart et al. 1993). Whole cell current-clamp recordings were obtained from CA1 pyramidal neurons in slices using an Axoclamp 2B (Axon Instruments) amplifier in the discontinuous current-clamp mode after balancing the bridge and neutralizing the capacitance. Voltage-clamp recordings from cultured neurons, except for current-voltage (I-V) plots, were obtained at a holding potential of −65 mV. Voltage-clamp recordings were not obtained from hippocampal slices in this study. Current-clamp recordings from cultured neurons and CA1 pyramidal neurons in slices were obtained at the neuron’s resting membrane potential. All voltages were corrected for calculated junction potentials.

SOLUTIONS. Recordings from cultured hippocampal neurons were made at room temperature with an extracellular solution containing (in mM) 140 NaCl, 5 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 d-glucose, and 10 HEPES (pH 7.40). The recording chamber holding cultured neurons was continuously perfused with extracellular solution at 0.5 ml/min. For all current-clamp recordings, 140 mM CsCl replaced the K gluconate in the pipette solution.

Mg²⁺ was omitted from the extracellular solution to induce neuronal bursting as described previously (Goodkin et al. 2005; Mangan and Kapur 2004). Ten micromolar d-serine was added to the extracellular solution in the bursting experiments to saturate the glycine binding site on the N-methyl-d-aspartate receptor (NMDAR) (Chen et al. 2003; Matsui et al. 1995).

For current-clamp recordings from cultured neurons and CA1 pyramidal neurons in slices, patch pipettes were filled with a solution containing (in mM) 140 K gluconate, 4 NaCl, 0.5 CaCl₂, 5 EGTA, 0.5 Na₃ GTP, 2 Mg ATP, and 10 HEPES (pH 7.20). For most voltage-clamp recordings, 140 mM CsCl replaced the K gluconate in the pipette solution.

For gramicidin perforated-patch recordings, 140 mM KCl replaced the K gluconate in the internal solution, which also contained 0.1 mg/ml gramicidin (Ye 2000). Cs⁺ was not used because the K⁺-Cl⁻ cotransporter cannot use Cs⁺ (Kakazu et al. 2000). The use of a 145 mM Cl⁻ containing internal solution allowed us to detect a spontaneous rupture of the membrane under the electrode because the Cl⁻ equilibrium potential will approach 0 mV. We confirmed this change experimentally by intentionally rupturing the membrane and measuring the glycine current reversal potential.

We used a high performance liquid chromatography/tandem mass spectrometry assay to measure glycine concentrations in our ACSF. The basal glycine and β-alanine concentrations in the ACSF were <1 μM, the lowest detectable concentration. Thus basal glycine and β-alanine concentrations in our ACSF are insufficient to activate GlyRs (Thio and Zhang 2006; Thio et al. 2003). We note that GlyR activation requires taurine concentrations >50 μM (Thio and Zhang 2006; Thio et al. 2003).

DRUG APPLICATION. Drug containing solutions were applied to cultured neurons using a multibarrel, gravity-driven, flow tube system as previously described (Thio et al. 2003). Drugs were applied to hippocampal slices by adding them to the perfusate.

DATA ANALYSIS. Data from cultured neurons were filtered with a 1- or 2-kHz low-pass filter and digitized at 5 or 10 kHz using pCLAMP 9 (Axon Instruments). Data from CA1 pyramidal neurons in slices were low-pass filtered at 3 kHz and digitized at 50 kHz using pCLAMP 9. All data were analyzed using pCLAMP 9, Origin 7 (OriginLab, Northampton, MA), and GraphPad (GraphPad Software, San Diego, CA). Glycine I-V plots were obtained by applying voltage ramps at 0.1 V/s during steady-state glycine currents or by holding neurons at various potentials and applying glycine.

The EC₅₀ for the glycine steady-state current and the IC₅₀ for strychnine inhibition of the glycine steady-state current were determined by obtaining complete dose–response curves from single neurons. The dose–response curve for the glycine current in each neuron was fit to the logistic equation

\[
R(\text{glycine}) = \frac{R_{\text{max}} \times [\text{glycine}]^n}{E_{C50}^n + [\text{glycine}]^n}
\]

where \(R(\text{glycine})\) is the response to a given \([\text{glycine}]\), \(R_{\text{max}}\) is the response to a saturating \([\text{glycine}]\), and \(n\) is the Hill coefficient. Strychnine dose–response curves were fit to the logistic equation

\[
R(\text{strychnine}) = \frac{R_{\text{peak}} \times IC_{50}^{\text{n}}}{IC_{50}^{n} + [\text{strychnine}]^{n}}
\]

where \(R(\text{strychnine})\) is the response to a given \([\text{glycine}]\) in the presence of a given \([\text{strychnine}]\), \(R_{\text{peak}}\) is the response to the same \([\text{glycine}]\) in the absence of strychnine, and \(n\) is the Hill coefficient. Fits were obtained using the Levenberg-Marquardt algorithm.

Neurons were subjected to 700-ms hyperpolarizing current steps to determine input resistance and 700-ms depolarizing current steps to generate action potentials. Cultured neurons were subjected to −40- to +30-pA current steps in 10-pA increments, whereas CA1 pyramidal neurons in slices were subjected to −400- to +100-pA current steps in 100-pA increments. Input resistance was equal to the slope of the line obtained by plotting the membrane voltage 600 ms after the start of a hyperpolarizing current step versus the magnitude of the current step. Action potential parameters assessed included amplitude, duration, maximum rate of rise, threshold, and interval between action potentials. Amplitude was the difference between the peak voltage and the resting potential. Duration was the width at half-amplitude. The maximum rate of rise was the maximum of the first derivative (dV/dt) of the rising phase. Threshold was the voltage during the rising phase at which dV/dt reached 2% of its maximum (Khalig and Raman 2006). The interval between action potentials was the time between action potential peaks. The rheobase for CA1 pyramidal neurons was determined by injecting 200-ms depolarizing current steps in 5-pA increments. The smallest current step resulting in the generation of an action potential was defined as the rheobase.

1000 (Vibratome, St. Louis, MO). Slices were transferred to a holding chamber filled with room temperature artificial cerebrospinal fluid (ACSF) saturated with 95% O₂-5% CO₂, and containing (in mM) 125 NaCl, 26 NaHCO₃, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, and 10 d-glucose. Slices were allowed to recover for at least 1 h before recording.
Neuronal bursts were defined as spike or action potential trains in which the time between successive spikes was less than or equal to twice the average interval between successive spikes in a burst. This definition identified bursts as a series of spikes superimposed on a depolarizing plateau (Goodkin et al. 2005; Mangan and Kapur 2004; Segal and Furshpan 1990). Burst duration refers to the time between the first and last spike in a burst. Spikes were detected using the threshold mode for event detection in pCLAMP 9 with a threshold of 0 mV. Overall spike frequency was the total number of spikes that occurred during a recording period divided by the recording time.

All data are presented as the mean ± SE. Comparisons were made using a Student’s t-test, a one-way ANOVA followed by Tukey’s post hoc comparison of means, a two-way ANOVA, or a repeated-measures two-way ANOVA followed by Bonferroni post hoc analysis. Statistical significance was set at \( P < 0.05 \).

**Chemicals**

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest grade available.

**RESULTS**

**Extracellular Zn\(^{2+}\) (1 \( \mu \)M) potentiates steady-state glycine currents**

Glycine evokes dose-dependent, strychnine-sensitive, chloride currents mediated by \( \alpha_2 \)-containing GlyRs in cultured embryonic mouse hippocampal neurons (Thio et al. 2003). In this preparation, extracellular Zn\(^{2+}\) concentrations near 1 \( \mu \)M at least double the peak current elicited by sub saturating concentrations of glycine (Thio and Zhang 2006). We first sought to show that low micromolar extracellular Zn\(^{2+}\) concentrations also augment steady-state glycine currents because our goal was to examine whether potentiating extracellular Zn\(^{2+}\) concentrations enhance the inhibitory effects of sustained GlyR activation.

In a nominally Zn\(^{2+}\)-free extracellular solution, glycine evoked a dose-dependent, strychnine-sensitive, steady-state current (Fig. 1). Zn\(^{2+}\) (1 \( \mu \)M) increased the amplitude of the peak and steady-state current evoked by 20 \( \mu \)M glycine (EC\(_5\) for the peak current, EC\(_{25}\) for the steady-state current in the nominal absence of Zn\(^{2+}\)) by 110 ± 18 (\( n = 10 \)) and 73 ± 32% (\( n = 10 \)) (Fig. 1, A and B). In comparison, it only increased the amplitude of the peak and steady-state current evoked by 60 \( \mu \)M glycine (EC\(_{50}\) for the peak current, EC\(_{70}\)) for the steady-state current in the nominal absence of Zn\(^{2+}\)) by 25 ± 5 (\( n = 10 \)) and 16 ± 13% (\( n = 10 \)) (Fig. 1, A and B). To determine if 1 \( \mu \)M Zn\(^{2+}\) altered the EC\(_{50}\) and the N for the glycine steady-state current, complete dose–response curves were obtained for the steady-state glycine current in single neurons. The EC\(_{50}\) for the glycine steady-state current decreased from 35 ± 1 \( \mu \)M (\( n = 6 \)) in the nominal absence of Zn\(^{2+}\) to 23 ± 3 \( \mu \)M (\( n = 7 \), \( P < 0.01 \) by Student’s t-test) in 1 \( \mu \)M Zn\(^{2+}\). N decreased from 1.5 ± 0.1 (\( n = 6 \)) in the nominal absence of Zn\(^{2+}\) to 1.1 ± 0.2 (\( n = 7 \), \( P < 0.05 \) by Student’s t-test) in 1 \( \mu \)M Zn\(^{2+}\). The data from all neurons were combined and are shown in Fig. 1.
by normalizing the amplitude of the steady-state current for each glycine concentration to that evoked by 300 μM glycine in the same cell (Fig. 1C). The normalized dose–response curves in the nominal absence of Zn²⁺ and in 1 μM Zn²⁺ differed (see Fig. 1C for results of 2-way repeated-measures ANOVA). We used the same approach to determine whether 1 μM Zn²⁺ altered the inhibition of the steady-state glycine current by strychnine. We examined the inhibition of the steady-state current evoked by an EC₅₀ glycine concentration in the nominal absence of Zn²⁺ and in 1 μM Zn²⁺. The IC₅₀ for strychnine increased from 13 ± 3 nM (n = 5) in the nominal absence of Zn²⁺ to 30 ± 3 nM (n = 6, P < 0.01 by Student’s t-test) in 1 μM Zn²⁺, but N did not change. N was 0.8 ± 0.1 (n = 5) in the nominal absence of Zn²⁺ and 0.9 ± 0.1 (n = 6) in 1 μM Zn²⁺. The normalized glycine dose–response curves in the nominal absence of Zn²⁺ and in 1 μM Zn²⁺ differed (Fig. 1D, see legend for results of 2-way repeated-measures ANOVA). Cl⁻ mediates steady-state glycine currents because their reversal potentials changed as expected with changes in intracellular Cl⁻ concentration (data not shown). Thus 1 μM Zn²⁺ potentiated steady-state glycine currents mediated by GlyRs.

**Extracellular Zn²⁺ (1 μM) enhances the inhibition of action potential firing by sustained GlyR activation**

Next, we used gramicidin perforated patch recordings to estimate the intracellular Cl⁻ in the hippocampal neurons in our preparation. Using the Nernst equation, the reversal potentials for the 20 μM glycine steady-state current in 1 μM Zn²⁺ yielded an intracellular Cl⁻ concentration of 14 ± 2 mM (n = 6) at 7–9 days and 8 ± 1 mM (n = 5, P < 0.05 compared with 7–9 days by Student’s t-test) at 13 days in culture as reported previously (Kuner and Augustine 2000). The neurons in all the remaining experiments were cultured for ≥9 days, and 65% were cultured for >10 days. Thus we used the K gluconate internal solution, containing 5 mM Cl⁻, for all remaining experiments, which were performed using the standard whole cell configuration.

We used evoked action potentials to determine if 1 μM Zn²⁺ can potentiate the inhibitory effects of sustained GlyR activation on neuronal excitability under normal conditions. A 700-ms depolarizing current step readily evoked a train of action potentials in hippocampal neurons in the nominal absence of Zn²⁺ and glycine (Figs. 2A and 3A). Zn²⁺ (1 μM) alone produced a small but not significant decrease in action potential firing (Figs. 2, A and B, and 3, A and B). A sustained 20 μM glycine application produced a small but not significant decrease in the number of action potentials elicited by a 20- and 30-pA depolarizing step in the nominal absence of Zn²⁺ (Fig. 2, A and B). However, the same glycine application significantly decreased the number of action potentials elicited by the same steps in 1 μM Zn²⁺ (Fig. 2, A and B), an effect blocked by 1 μM strychnine (data not shown). In contrast, a sustained 60 μM glycine application in the nominal absence of Zn²⁺ markedly decreased the number of action potentials elicited during a 20- and 30-pA depolarizing step (Fig. 3, A and B). Adding 1 μM Zn²⁺ did not alter the effects of 60 μM glycine on action potential firing during a 20-pA depolarizing step in part because it could not further reduce firing in some neurons. Notably, a 20-pA depolarizing step during a sustained 60 μM glycine application in the nominal absence of Zn²⁺ evoked no action potentials in four of seven cells. However, only two of seven neurons fired no action potentials during a 30-pA depolarizing step while applying 60 μM glycine in the nominal absence of Zn²⁺. Thus 1 μM Zn²⁺ enhanced the depression of action potential firing induced by 20 μM but not 60 μM glycine as expected from its effects on the steady-state currents elicited by these glycine concentrations.

A decrease in input resistance accompanied the depression in action potential firing induced by glycine (Figs. 2C and 3C). In parallel with its effects on action potential firing, 20 μM glycine decreased neuronal input resistance by 10 ± 3% (100 ± 30 MΩ) in 9/15 neurons in the nominal absence of Zn²⁺. The small and variable decrease induced by 20 μM glycine in the nominal absence of Zn²⁺ led to no significant change in the input resistance overall (Fig. 2C). However, 20 μM glycine decreased neuronal input resistance by 29 ± 8% (n = 10) in 1 μM Zn²⁺ (Fig. 2C). This effect was blocked by 1 μM strychnine (data not shown). As expected from its effects on action potential firing, 1 μM Zn²⁺ did not significantly potentiate the decrease in neuronal input resistance induced by 60 μM glycine (Fig. 3C). Glycine (60 μM) decreased neuronal input resistance by 46 ± 7% (n = 9) in the nominal absence of Zn²⁺ and by 53 ± 7% (n = 9) in 1 μM Zn²⁺. Zn²⁺ (1 μM) had no effect on input resistance by itself (Figs. 2C and 3C).

No change in the resting membrane potential accompanied the glycine-induced depression in action potential firing. Neither 20 nor 60 μM glycine altered the resting membrane potential in the nominal absence of Zn²⁺ or in 1 μM Zn²⁺ (Figs. 2D and 3D). This lack of change in the resting membrane potential even with glycine and Zn²⁺ combinations that decreased input resistance probably reflects the proximity of the resting membrane potential to the glycine reversal potential, which was −64 ± 2 mV (n = 3), using the K gluconate internal solution. This potential is more positive than the Cl⁻ reversal potential, which was −90 mV under our conditions possibly because GlyRs have a permeability ratio for gluconate to Cl⁻ of 0.26 (Fatima-Shad and Barry 1993). In addition, intracellular Cl⁻ may accumulate with low internal Cl⁻ concentrations because of the K⁺–Cl⁻ cotransporter (DeFazio et al. 2000). Zn²⁺ (1 μM) alone had no effect on the resting membrane potential (Figs. 2D and 3D).

No change in action potential properties accompanied the glycine-induced decrease in action potential firing. Sustained 20 μM glycine did not alter action potential amplitude, duration, maximum rate of rise, threshold, and interval in the nominal absence of Zn²⁺ or in 1 μM Zn²⁺. Figure 2, E–H, shows amplitudes, durations, thresholds, and intervals for the first five action potentials evoked by a 30-pA depolarizing step. We typically did not observe more than four action potentials when applying 20 μM glycine + 1 μM Zn²⁺. We could not perform a similar analysis for the action potentials evoked by a 20-pA depolarizing step in 20 μM glycine + 1 μM Zn²⁺ because few neurons fired action potentials. For the same reason, we could not analyze the properties of the action potentials evoked by a 20- or 30-pA depolarizing step in 60 μM glycine + 1 μM Zn²⁺. Zn²⁺ (1 μM) alone had no significant effect on action potential properties (Fig. 2, E–H).
Zn\(^{2+}\) (1 μM) enhances the inhibition of hyperexcitability by sustained GlyR activation

Next, we examined whether 1 μM Zn\(^{2+}\) potentiates the inhibitory effects of sustained GlyR activation on neuronal firing under hyperexcitable conditions. We elected to induce hyperexcitability by omitting Mg\(^{2+}\) from the extracellular solution because sustained GlyR activation inhibits epileptiform activity in this model (Kirchner et al. 2003). This model has provided an explanation for phenomena observed during seizures in humans and animals such as the development of benzodiazepine resistance during a seizure (Goodkin et al. 2005). In addition, the model is robust, depends on a network, and shares mechanistic properties with other models for acutely inducing epileptiform activity in vitro (Mangan and Kapur 2004; Tian et al. 2005).

In 1 mM extracellular Mg\(^{2+}\), cultured hippocampal neurons occasionally fire action potentials spontaneously. When exposed to a nominally Mg\(^{2+}\)-free extracellular solution, neurons fired recurrent bursts of action potentials, which in 81% (36/44) of neurons were superimposed on a prolonged depolarization resembling a paroxysmal depolarizing shift (Fig. 4A) (Goodkin et al. 2005; Mangan and Kapur 2004; Segal and Furshpan 1990). Burst frequency, burst duration, and overall spike frequency were similar in the nominal absence of Zn\(^{2+}\) and in 1 μM Zn\(^{2+}\) (Figs. 4, B and C, and 5, B and C). Thus 1 μM extracellular Zn\(^{2+}\) did not alter the neuronal bursting induced by omitting extracellular Mg\(^{2+}\).
We examined the effect of 200 μM taurine and glycine concentrations in the nominal absence of Zn2+ (Thio et al. 2001), and the estimated basal extracellular glycine concentration is 2–10 μM in the rat cortex (Matsui et al. 1995; Westergren et al. 1994). Thus Zn2+ potentiation of GlyRs may help modulate neuronal excitability in the hippocampus if GlyRs are tonically active as prior studies suggest (Mori et al. 2002; Zhang et al. 2007).

We examined the effect of strychnine and tricine on action potential firing by CA1 pyramidal neurons to determine if basal Zn2+ and endogenous GlyR agonist concentrations in hippocampal slices modulate neuronal excitability. A 700-ms depolarizing 100-pA step readily evoked a train of 18 ± 2 (n = 7) action potentials in CA1 pyramidal neurons (Fig. 6A). Although applying 1 μM strychnine or 10 mM tricine individually did not alter the number of action potentials elicited by a depolarizing 100-pA step, co-applying 1 μM strychnine and 10 mM tricine slightly but significantly increased the number of action potentials fired by 1.3 ± 0.3 (n = 7; Fig. 6A and C). The number of action potentials fired during the co-application of strychnine and tricine increased in six of the seven neurons examined with the remaining neuron showing no change. In all seven neurons, a small but significant 12 ± 1 MΩ increase in input resistance accompanied this increase in action potential firing (Fig. 6D). CA1 neurons under control conditions had an input resistance of 200 ± 32 MΩ (n = 7), which tricine and

Co-applying strychnine and tricine decreases the rheobase of hippocampal CA1 pyramidal neurons

Zn2+ potentiated the inhibitory effect of sustained GlyR activation on evoked action potentials under normal conditions and on neuronal bursting induced by omitting Mg2+. This effect occurred at Zn2+ and glycine concentrations that may be present endogenously. The estimated basal extracellular Zn2+ concentration in the hippocampus is 2–3 μM (Kay 2003; Li et al. 2001), and the estimated basal extracellular glycine concentration is 2–10 μM in the rat cortex (Matsui et al. 1995; Westergren et al. 1994). Thus Zn2+ potentiation of GlyRs may help modulate neuronal excitability in the hippocampus if GlyRs are tonically active as prior studies suggest (Mori et al. 2002; Zhang et al. 2007).

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strychnine alone did not alter (Fig. 6D). Next, we examined whether co-applying 1 μM strychnine and 10 mM tricine altered the rheobase, the minimum 200-ms depolarizing current required to elicit an action potential. Co-applying strychnine and tricine produced a small but significant decrease in the rheobase in all seven neurons examined (Fig. 6, B and E). The rheobase under control conditions was 31 ± 5 pA (n = 7), which strychnine and tricine alone did not alter (Fig. 6, B and E). Tricine and strychnine when applied alone or simultaneously did not alter action potential amplitude (110 ± 7 mV, n = 5, in control), duration (2.0 ± 0.2 ms, n = 5, in control), threshold (-55 ± 2 mV, n = 5, in control), or maximum rate of rise (440 ± 16 V/s, n = 5, in control). Thus co-applying tricine and strychnine slightly increased neuronal excitability in CA1 hippocampal pyramidal neurons.

**Discussion**

One finding of this study is that a potentiating extracellular Zn²⁺ concentration augments the inhibitory effects of sustained GlyR activation by an EC₂⁵ glycine concentration under normal and hyperexcitable conditions. This finding in cultured hippocampal neurons raises the possibility that Zn²⁺ enhances the inhibitory effect of tonically active GlyRs in hippocampal slices. Accordingly, action potentials in CA1 pyramidal neurons are slightly easier to elicit in hippocampal slices bathed in a nominally Zn²⁺-free solution, in a nominally Mg²⁺- and Zn²⁺-free solution, or in a nominally Mg²⁺-free solution that contains 1 μM Zn²⁺. Bars above traces indicate changes in extracellular Mg²⁺, glycine, and strychnine concentrations. Bottom trace in middle right panel shows indicated portion of top trace at an expanded time scale. Traces under each heading are from different neurons except for traces in the middle left and bottom left panels, which come from the same neuron. **B**: Zn²⁺ enhanced inhibitory effects of sustained 20 μM glycine applications on neuronal bursting induced by omitting extracellular Mg²⁺. Mean burst frequency, burst duration, and spike frequency in the nominal absence of Zn²⁺ (open bars, n = 13) or in 1 μM Zn²⁺ (filled bars, n = 15) and in the absence (left pair of bars) or presence of 20 μM glycine (right pair of bars). C: Zn²⁺ did not enhance inhibitory effects of sustained 60 μM glycine applications on neuronal bursting induced by omitting Mg²⁺. Mean burst frequency, burst duration, and spike frequency in the nominal absence of Zn²⁺ (open bars, n = 13) or in 1 μM Zn²⁺ (filled bars, n = 24) and in the absence (left pair of bars) or presence of 60 μM glycine (right pair of bars). D: strychnine prevented inhibition of neuronal bursting by sustained 20 μM glycine applications in 1 μM Zn²⁺. Mean burst frequency (top) and mean spike frequency (bottom) in control (open bars, n = 5) or in 1 μM strychnine (closed bars, n = 5) and in the absence (left pair of bars) or presence of 20 μM glycine (right pair of bars). Lines above bars in B–D indicate significantly different pairs; other pairs did not differ. *P < 0.05, **P < 0.01, and ***P < 0.001 by 1-way ANOVA followed by Tukey’s post hoc comparison of means.

![Diagram](http://jn.physiology.org/)

**Fig. 4.** Extracellular Zn²⁺ (1 μM) enhances GlyR-mediated inhibition of hippocampal neuronal hyperexcitability induced by omitting extracellular Mg²⁺. **A**: voltage traces from cultured hippocampal neurons bathed in a nominally Zn²⁺-free solution, in 1 μM Zn²⁺, in a nominally Mg²⁺- and Zn²⁺-free solution, or in a nominally Mg²⁺-free solution that contains 1 μM Zn²⁺. Bars above traces indicate changes in extracellular Mg²⁺, glycine, and strychnine concentrations. Bottom trace in middle right panel shows indicated portion of top trace at an expanded time scale. Traces under each heading are from different neurons except for traces in the middle left and bottom left panels, which come from the same neuron. **B**: Zn²⁺ enhanced inhibitory effects of sustained 20 μM glycine applications on neuronal bursting induced by omitting extracellular Mg²⁺. Mean burst frequency, burst duration, and spike frequency in the nominal absence of Zn²⁺ (open bars, n = 13) or in 1 μM Zn²⁺ (filled bars, n = 15) and in the absence (left pair of bars) or presence of 20 μM glycine (right pair of bars). **C**: Zn²⁺ did not enhance inhibitory effects of sustained 60 μM glycine applications on neuronal bursting induced by omitting Mg²⁺. Mean burst frequency, burst duration, and spike frequency in the nominal absence of Zn²⁺ (open bars, n = 13) or in 1 μM Zn²⁺ (filled bars, n = 24) and in the absence (left pair of bars) or presence of 60 μM glycine (right pair of bars). **D**: strychnine prevented inhibition of neuronal bursting by sustained 20 μM glycine applications in 1 μM Zn²⁺. Mean burst frequency (top) and mean spike frequency (bottom) in control (open bars, n = 5) or in 1 μM strychnine (closed bars, n = 5) and in the absence (left pair of bars) or presence of 20 μM glycine (right pair of bars). Lines above bars in B–D indicate significantly different pairs; other pairs did not differ. *P < 0.05, **P < 0.01, and ***P < 0.001 by 1-way ANOVA followed by Tukey’s post hoc comparison of means.
Effects occurred with either an EC70 glycine concentration inducing by the absence of extracellular Mg2+. Sustained GlyR activation reduced spontaneous neuronal burst activity in cultured hippocampal neurons as observed previously in hippocampal conditions inhibited action potential firing in cultured hippocampal neurons as observed previously in hippocampal conditions inhibited action potential firing in cultured hippocampal neurons (Segal and Furshpan 1990). These results are consistent with prior findings indicating that sustained GlyR activation has anticonvulsive properties. Thus GlyR activation inhibits action potential firing under normal conditions when it produces a sufficient shunt.

The neuronal bursting induced by omitting Mg2+ from the extracellular solution is partly dependent on N-methyl-D-aspartate receptor (NMDAR) activation. NMDARs are among the many other ligand-gated ion channels, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPArs) and GABArRs, modulated by Zn2+. Many other ligand-gated ion channels, including AMPArs and GABArRs require higher concentrations. Although Zn2+ effectively inhibits synaptic NMDARs, Zn2+ alone did not prevent neuronal bursting. This result is not surprising because others using this model did not attempt to reduce extracellular Zn2+ concentrations to submicromolar levels (Goodkin et al. 2005; Mangan and Kapur 2004; Segal and Furshpan 1990). The extracellular solutions used in these earlier studies likely contained several hundred nanomolar Zn2+ as found in most solutions (Thio and Zhang 2006; Wilkins and Smart 2002). In the nominal absence of Zn2+, a sustained application of 20 μM glycine had little effect on action potential firing under normal conditions. The same glycine concentration in 1 μM Zn2+ markedly reduced action potential firing. The effects of 20 μM glycine on action potential firing in the nominal absence of Zn2+ and in 1 μM Zn2+ paralleled its effects on input resistance. This effect of 1 μM Zn2+ probably results from Zn2+ potentiation of GlyR mediated responses, which causes a greater decrease in input resistance and shunting. The observation that 60 μM glycine decreases action potential firing and input resistance in the nominal absence of Zn2+ supports this conclusion. Conceivably, Zn2+ modulation of voltage-gated ion channels could have contributed to the reduction in action potential firing by 20 μM glycine in 1 μM extracellular Zn2+. However, Zn2+ modulation of voltage-gated ion channels requires higher Zn2+ concentrations (Mathie et al. 2006). In addition, Zn2+ augmented the inhibition of action potential firing by 20 μM glycine without significantly altering the resting membrane potential or action potential properties. Thus GlyR activation inhibits action potential firing under normal conditions when it produces a sufficient shunt.

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\text{Zn}^{2+} \text{ potentiates the inhibitory effects of sustained hippocampal GlyR activation in culture}
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The neuronal bursting induced by omitting Mg2+ from the extracellular solution is partly dependent on N-methyl-D-aspartate receptor (NMDAR) activation. NMDARs are among the many other ligand-gated ion channels, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPArs) and GABArRs, modulated by Zn2+ (Celentano et al. 1991; Lin et al. 2001; Paolletti et al. 1997). NMDARs and GlyRs are similar in that endogenous Zn2+ concentrations in the hippocampus are sufficient to modulate both, whereas AMPARs and GABArRs require higher concentrations. Although Zn2+ effectively inhibits synaptic NMDARs, Zn2+ alone did not prevent neuronal bursting. This result is not surprising because others using this model did not attempt to reduce extracellular Zn2+ concentrations to submicromolar levels (Goodkin et al. 2005; Mangan and Kapur 2004; Segal and Furshpan 1990). The extracellular solutions used in these earlier studies likely contained several hundred nanomolar Zn2+ as found in most solutions (Thio and Zhang 2006; Wilkins and Smart 2002). In the nominal absence of Zn2+, a sustained application of 20 μM glycine had little effect on action potential firing. However, 1 μM Zn2+ together with 20 μM glycine was very effective in reducing neuronal bursting. Zn2+ (1 μM) also augmented the inhibitory
FIG. 6. Co-applying strychnine and tricine increases the excitability of CA1 pyramidal neurons in hippocampal slices. A: voltage traces from 1 CA1 pyramidal neuron subjected to 700-ms current steps from −300 to +100 pA in 100-pA increments. Traces were obtained in control artificial cerebrospinal fluid (ACSF) and in ACSF containing 1 μM strychnine, 10 mM tricine, or 1 μM strychnine + 10 mM tricine as indicated. Number of action potentials elicited by a 100-pA depolarizing step was 28 in control ACSF and in ACSF containing 1 μM strychnine or 10 mM tricine. The same depolarizing step evoked 29 action potentials in ACSF containing 1 μM strychnine + 10 mM tricine. B: voltage traces from another CA1 pyramidal neuron used to determine the rheobase for action potential firing. The neuron was subjected to a series of 200-ms depolarizing current steps in 5-pA increments as indicated. Rheobase was defined as smallest current injection evoking an action potential. Traces were obtained under the same experimental conditions as in A. Resting membrane of the neuron was −67 mV. C–E: co-applying strychnine and tricine increased number of action potentials evoked by a 100-pA depolarizing step (C), increased input resistance (D), and decreased rheobase (E). Bars in C show mean difference between number of action potentials evoked by a 100-pA depolarizing step in 1 μM strychnine (n = 5), 10 mM tricine (n = 5), or 1 μM strychnine + 10 mM tricine (n = 7) containing ACSF and control ACSF. Bars in D show mean difference between input resistance in each experimental condition and control ACSF. Bars in E show mean difference between rheobase in each experimental condition and control ACSF. Lines above bars in C–E indicate significantly different pairs; other pairs did not differ. *P < 0.05, **P < 0.01, and ***P < 0.001 by 1-way ANOVA followed by Tukey’s post hoc comparison of means.
effects of 200 μM taurine on neuronal bursting. Our results suggest that endogenous Zn²⁺ and GlyR agonist concentrations cannot significantly depress neuronal hyperexcitability alone, but together they can. We have previously hypothesized that Zn²⁺ may be especially important if taurine is the endogenous agonist (Mori et al. 2002) because it may be necessary for GlyR activation by taurine (Thio and Zhang 2006). These findings may account in part for the increased seizure susceptibility of rodents treated with a Zn²⁺ chelator (Dominguez et al. 2003), fed a Zn²⁺-deficient diet (Takeda et al. 2003) or lacking vesicular Zn²⁺ because of defective Zn²⁺ transport (Cole et al. 2000).

**Zn²⁺ enhances tonic GlyR activation by endogenous agonists in hippocampal slices**

Co-applying strychnine and tricine slightly increased the excitability of CA1 pyramidal neurons in hippocampal slices by decreasing the rheobase for action potential firing. As expected from the results in culture, a slight increase in input resistance accompanied the decrease in the rheobase. These observations are consistent with the small tonic GlyR-mediated currents in hippocampal slices others have found (Mori et al. 2002; Zhang et al. 2007). Prior studies indicate that tonic GlyR activation induced by blocking glycine transport modulates hippocampal neuronal function (Song et al. 2006; Zhang et al. 2007). Our results suggest that tonic GlyR activation occurs in the hippocampus and modulates neuronal excitability even without adding a glycine transport inhibitor. They further suggest that the concentration of the endogenous agonist in our slices is low enough to allow Zn²⁺ to significantly potentiate its effects. Our experiments do not identify which GlyR agonist is responsible for our findings. They also do not address whether endogenous Zn²⁺ concentrations in our slices are sufficient to potentiate GlyRs because our ACSF contains 800 nM Zn²⁺, which originates from impurities in the NaCl (Thio and Zhang 2006). We are uncertain why neither strychnine nor tricine alone had a similar effect. However, strychnine alone may have been ineffective because it is a more potent inhibitor of glycine currents in the nominal absence of Zn²⁺ than in 1 μM Zn²⁺ (Fig. 1D). Tricine alone may have been ineffective because the effect Zn²⁺ has on other ion channels, such as inhibiting NMDARs (Paoletti et al. 1997), may obscure the loss of tonic GlyR activation on action potential firing. Nevertheless, our results suggest that Zn²⁺ and GlyRs may have an important role in tonically modulating neuronal excitability and are potential targets for neuropsychiatric drugs such as anticonvulsants.

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