Zinc-Induced Augmentation of Excitatory Synaptic Currents and Glutamate Receptor Responses in Hippocampal CA3 Neurons

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Received 19 September 2000; accepted in final form 8 December 2000

INTRODUCTION

Zinc, a heavy-metal, divalent cation, has long been implicated in a myriad of cellular processes, ranging from interactions with zinc-finger proteins and protein kinases to modulation of ion channels (as reviewed in Smart et al. 1994). In the CNS, zinc localizes to numerous regions, including the cerebrocortical and corticostriatal pathways (Perez-Clausell and Danscher 1985, 1986). Most notably, zinc has been shown to localize within the axon terminals of dentate granule cells. These mossy fibers synapse on the proximal dendrites of pyramidal neurons in region CA3 (Crawford and Connor 1972; Haug et al. 1971). It is at these axon terminals that zinc colocalizes with glutamate in synaptic vesicles and may be released into the synaptic cleft together with glutamate (Assaf and Chung 1984; Cole et al. 1999; Howell et al. 1984; Wenzel et al. 1997). Despite continuing studies for decades, however, precise determination of zinc’s physiological role at these mossy fiber synapses and in the CNS in general remains elusive. This study was conducted in an attempt to determine how zinc co-release may impact excitatory neurotransmission and glutamate receptor function in CA3 neurons during both normal and pathological states.

Zinc affects multiple voltage-gated and ligand-gated ion channels. Most noteworthy are the GABA A , the N-methyl-D-aspartate (NMDA), and the a-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) receptor–mediated currents (AMPAR currents) that are involved in modulating glutamate-evoked currents, suggesting that zinc may potentiate AMPAR currents by inhibiting AMPAR desensitization. Based on the results of the present study, we hypothesize that zinc is a powerful modulator of both excitatory synaptic transmission and glutamate-evoked currents at physiologically relevant concentrations. This modulatory role played by zinc may be a significant factor in enhancing excitatory neurotransmission and could significantly regulate function at the mossy fiber-CA3 synapse.

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1984), this heavy metal’s presence in these excitatory synapses may be of great physiological import in the modulation of function at MF-CA3 synapses.

The majority of electrophysiological studies investigating zinc’s effects on AMPARs have been conducted employing bath application of zinc onto transfected oocytes, HEK cells, or hippocampal cultures during whole cell patch-clamp recordings (for review, see Smart et al. 1994). In these studies, zinc has been demonstrated to have concentration-dependent effects on AMPAR currents. Mayer et al. (1989) demonstrated that, at concentrations <300 μM, zinc potentiated AMPAR currents, whereas at higher concentrations (>500 μM), zinc inhibited AMPAR currents. AMPARs, however, are characterized by ultrarapid desensitization (cf. Zorumski and Thio 1992), and it is impossible to change solutions rapidly enough using whole cell patch techniques to determine the peak of the AMPA receptor currents. In addition, AMPARs on these cultured neurons and transfected cells would not be exposed to zinc under physiological conditions, where extracellular zinc concentrations may reach 100–300 μM during normal functioning of the MF-CA3 synapse (Frederickson et al. 1983). Although a recent study describes effects of synaptically released zinc on postsynaptic NMDA receptors at MF synapses (Vogt et al. 2000), to date there have been no published reports that assess zinc’s effects at physiologically relevant concentrations on synaptic miniature excitatory postsynaptic currents (mEPSCs) or on native perisomatic AMPAR currents in CA3 neurons. Therefore to accomplish these goals, in the present study, whole cell slice patch techniques examining zinc’s effects on excitatory synaptic currents were combined with ultrafast solution switching techniques to investigate zinc’s effects on glutamatergic synapses and glutamate receptors in perisomatic outside-out patches of CA3 neurons.

**Methods**

**Slice preparation**

Experiments were performed on 15- to 21-day-old male Sprague-Dawley rats. Rats were anesthetized and decapitated, and their brains were rapidly removed and placed into cold (4°C), oxygenated (95% O2-5% CO2) sucrose-based artificial cerebrospinal fluid (SACSF) containing (in mM) 130 NaCl, 3 KCl, 26 NaHCO3, 1.25 NaH2PO4, 10 glucose, 2 MgCl2, and 2 CaCl2. The brains were chilled for 3 min before being cut into slices with a vibratome (Lancer 1000, St. Louis, MO). For synaptic studies, 300–μm hippocampal-entorhinal-cortical (HEC) slices were cut as described previously (Rafiq et al. 1995), to maintain the maximal number of intact dentate granule cell–CA3 axons and synapses. The presence of intact axons has been shown to impact the frequency of mEPSCs (Staley and Mody 1991). Brains were hemisectioned and each side was glued, rostral-side up, onto a 12° agar ramp. HEC slices were then isolated by trimming away the cortical and thalamic tissue with a scalpel. For outside-out patch studies, 250-μm transverse slices were cut. All slices were immediately transferred to oxygenated artificial cerebrospinal fluid (ACSF) in a holding chamber containing (in mM) 130 NaCl, 3 KCl, 26 NaHCO3, 1.25 NaH2PO4, 10 glucose, 1.0 MgCl2, and 2.0 CaCl2. Slices were incubated at 32–33°C for >1 h prior to initiating experiments.

**Visualization**

Brain slices were placed in a recording chamber at room temperature and continually perfused with oxygenated ACSF at a rate of 5 ml/min. CA3 pyramidal neurons were visualized using video-enhanced infrared differential interference contrast microscopy with an Olympus BH-2 upright microscope (Olympus America, Melville, NY) fitted with Nomarski optics, an infrared filter, and a ×40 long working distance water-immersion lens.

**Electrodes and intracellular solutions**

Patch electrodes were pulled from thick-walled borosilicate capillary glass (WPI, Sarasota, FL) and filled with an intracellular pipette solution, comprised of either (in mM) 145 K-gluconate, 10 HEPES, 10 bis-(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA), and 5 MgATP (K-gluconate internal solution) or 145 CsCl, 10 HEPES, 10 BAPTA, and 5 MgATP (CsCl internal solution). Results using either intracellular solution were similar. Osmolarity was adjusted to 290 mOsm with sucrose, and pH was adjusted to 7.2 with CsOH or KOH. A Narishige PP-83 microelectrode two-stage puller was used to pull 4–6 MΩ electrodes for whole cell recordings and 8–12 MΩ electrodes for outside-out patch studies.

**Rapid application**

Fast application of agonist was performed as described by Jonas and colleagues (Jonas 1995). Application pipettes were fashioned by pulling theta glass (2 mm OD, 0.3 mm wall thickness, 0.1167 mm septum, Hilgenberg GMBH, Malsfeld, Germany) on a Narishige PP-83 electrode puller. A diamond scribe was then used to score the theta glass such that each barrel had a sharp, clean edge and a diameter ranging between 100 and 150 μm. The theta glass was mounted on a piezoelectric transducer (Burleigh LSS-3100, Burleigh Instruments, Fisher, NY) and power supply (Burleigh PZ-150 amplifier/drive). Either Clampex 6.0 or 7.0 (Axon Instruments, Foster City, CA) waveform protocols were used to deliver command potentials resulting in applications of 1-ms pulses of agonists to the excised patches. Agonists and antagonists were dissolved in a control Ringer solution containing (in mM) 135 NaCl, 5.4 KCl, 1.8 CaCl2, 1 M MgCl2, and 5 HEPES, pH adjusted to 7.2 with NaOH. 325 mOsm (Spruston et al. 1995). This prevented pH changes that would occur in normal HCO3- buffered ACSF solutions without gassing. For experiments measuring AMPAR currents, 30 μM D-2-amino-5-phosphonopentanoic acid (D-AP5) was added to both barrels, glycine was omitted, and agonist pulses were typically applied at 4-s intervals. In experiments in which NMDA receptor-mediated currents were isolated, responses were elicited at 10-s intervals and with 10 μM glycine and 5 μM CNQX added to both perfusion solutions. Each theta barrel was constructed with several fused silica tubes cemented in place, which in turn were connected to tubing, a solenoid valve, and solution reservoirs. One tube flowed to waste, also under solenoid control. This perfusion setup, coupled with an electronic solenoid switching system allowed the solutions exposed to the patch in both barrels to be exchanged with no cross contamination within about 10 s, enabling the glutamate and glutamate plus drug experiments to be conducted (see Figs. 3–8).

On excision of the outside-out patch, the tip of the patch electrode was positioned in the control stream, approximately 20 μm from the interface separating the control and drug streams. Typically, the patches yielded AMPAR currents between 10 and 500 pA and NMDA receptor–mediated currents between 5 and 70 pA. After completion of patch recording, patches were expelled, and the exchange time was measured by recording the tip potential across an interface between control solution and a 80% control/20% distilled H2O solution. The 20–80% exchange times typically ranged between 100 and 175 μs.

**Electrophysiology**

In the whole cell configuration, mEPSCs were isolated by the addition of 400 nM tetrodotoxin (TTX) and 10 μM bicuculline methiodide (BMI) to the ACSF. Recordings in both whole cell and outside-out patch configurations were performed with an Axo-
patch-1D (Axon Instruments, Foster City, CA) and filtered at 1–3 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). In the whole cell configuration, series resistances were <20 MΩ and checked frequently to ensure that they did not deviate. Cells in which series resistance was >20 MΩ or in which series resistance fluctuated were discarded. A series resistance compensation level of 80% was utilized in all experiments. Data were digitized at 44 kHz with a PCM device (Neurodata Instruments, New York, NY) before being stored on videotape. For excited patch experiments, currents were consecutively digitized at 10–20 kHz and recorded onto disk with pClamp software and a Digidata 1200 A/D board (Axon Instruments).

Data analysis

Whole cell voltage-clamp data were played back into Dempster (Strathclyde, Glasgow, UK) software with a sampling interval of 50 μs. Ten to 20-min periods of activity were reacquired using Dempster software in both control and zinc-exposed conditions (number of events typically 500–2,000 per epoch), and kinetic analyses performed using either Dempster or Mini analysis software (Synaptosoft, Leonia, NJ). For rapid application studies, Clampfit 6.0 (Axon Instruments) was used to average five or more consecutive traces to ascertain peak amplitude and trace kinetics. Curve fits were performed with SigmaPlot 2.0 (Jandel Scientific, San Rafael, CA), and statistical analyses were performed using SigmaStat 2.0 (Jandel Scientific), and values expressed in the text are means ± SE. The statistical significance was determined at a P < 0.05 value using the Student’s paired t-test for zinc effects, and Student’s unpaired t-test for group comparisons.

Reagents

All solutions were prepared fresh from stocks each day. ZnCl₂ was diluted from a pH 7.2 10-mM stock solution in 20 mM stock solution, which was prepared every 3–4 days. Buffering the ZnCl₂ stock solution and diluted from a pH 7.2 10-mM stock solution, which was prepared for zinc effects, and Student’s unpaired t-test for zinc effects, and Student’s unpaired t-test for group comparisons.

CA3 mEPSCs are augmented by zinc

Spontaneously occurring mEPSCs were recorded from 25 CA3 neurons using TTX to block action potentials and BMI to block GABAAergic neurotransmission. Similar to other reports (Capogna et al. 1996; McBain and Dingledine 1992), the frequency of these events was extremely low. In only seven of these neurons was the mEPSC frequency high enough for adequate analysis. Previous investigators have determined that the fast peak component of CA3 mEPSCs is mediated by the AMPA subtype of glutamate receptors rather than the NMDA and kainate subtypes (Henze et al. 1997; Jonas et al. 1993; McBain and Dingledine 1992). The mean, putatively AMPAR-mediated peak amplitude of these CA3 mEPSCs was 18.1 ± 1.0 (SE) pA, similar to the results of other investigators (Henze et al. 1997; Jonas et al. 1993). Mean rise and decay times were 0.54 ± 0.1 ms and 1.6 ± 0.3 ms, respectively. The mEPSC frequency of these seven cells with sufficiently high frequency ranged from 0.13 to 3.6 Hz (mean, 1.5 ± 0.4 Hz).

On bath application of 200 μM ZnCl₂, mEPSC amplitude increased in six of seven CA3 neurons recorded (Fig. 1, A and B). The median peak amplitude of the synaptic events, presumably mediated by AMPAR activation, increased significantly to 31.9 ± 3.2 pA during zinc application (P < 0.05, Fig. 2A), while the mean mEPSC frequency was not significantly increased (frequency, 2.3 ± 0.7, P > 0.05, Fig. 2D). No significant changes in mEPSC rise and decay kinetics were observed during zinc application (rise time, 0.58 ± 0.16 ms; decay time, 2.5 ± 0.5 ms; Fig. 2C) compared with controls (Fig. 2, B and C). During zinc application, the amplitude and frequency of the mEPSCs varied largely from minute to minute without a consistent increase in mEPSC amplitude or frequency during the first few minutes of zinc application. Rather, occasional bursts of transient, high-frequency mEPSC activity (20–40 Hz) were observed; these bursts typically lasted <1.0 min and occurred at random intervals (data not shown). During burst periods, large-amplitude mEPSCs (>75 pA) were often observed. Because of the rapid kinetics of mEPSCs (Fig. 1), overlapping events were extremely rare and therefore did not confound analyses even during burst periods. Excluding these periods of bursting activity from the analysis did not alter the finding that zinc significantly augmented mEPSC amplitudes in CA3 neurons.

Glutamate-evoked currents are potentiated by zinc

Zinc colocalizes with glutamate in synaptic vesicles and is co-released with glutamate on high-frequency stimulation of nerve terminals (Cole et al. 1999; Howell et al. 1984; Wenzel et al. 1997). Since zinc presumably augments the AMPAR compo-
ment of excitatory synaptic events in region CA3, rapid agonist application experiments on perisomatic CA3 glutamate receptors were performed to characterize further zinc’s effects on glutamate receptors expressed in these neurons. With this technique, concentrations of agonists and modulators are easily regulated, allowing for more controlled experimental conditions that approximate synaptic release of glutamate. A saturating glutamate concentration of 3 mM (Min et al. 1998; Spruston et al. 1995), as well as 10 μM glycine, was chosen because previous studies have shown that glutamate may achieve millimolar concentrations within the synaptic cleft of hippocampal neurons (Clements 1996; Clements et al. 1992; Henze et al. 1997).

Similar to reports by others, 1-ms pulses of 3 mM glutamate to outside-out patches elicited glutamate-evoked currents that consisted of fast and slow components (Colquhoun et al. 1992; Livsey et al. 1993; Spruston et al. 1995). An initial rapid, presumably AMPAR-mediated rise and decay comprised the first component and was followed by a much slower, presumably NMDA receptor–mediated decay phase. Only patch currents with rise times <1.0 ms were accepted. Decay times (10–90%) ranged between 3.6 and 17.0 ms and were best fit with the sum of two exponentials.

To mimic zinc’s concurrent release with glutamate at presynaptic terminals, zinc was included only in the drug barrel with glutamate. As noted previously, at lower concentrations (<300 μM), zinc has been shown to augment non-NMDA glutamate receptor–mediated currents while antagonizing NMDA receptor–mediated currents (Bresink et al. 1996; Mayer et al. 1989; Rassendren et al. 1990; Xie et al. 1993). As seen in Fig. 3, the addition of zinc (200 μM) together with glutamate reversibly potentiated the peak currents elicited by 1-ms pulses of 3 mM glutamate alone in five of eight excised patches. In the five patches that demonstrated zinc sensitivity, zinc (200 μM) significantly augmented glutamate-evoked current amplitudes to 143.8 ± 9.8% of control levels evoked by glutamate alone (P < 0.05). The remaining three patches showed no zinc sensitivity, i.e., were neither potentiated nor depressed by zinc coapplication. This existence of dual glutamate receptor populations, one sensitive and one insensitive to zinc potentiation, has previously been reported by other investigators (Mayer et al. 1989; Rassendren et al. 1990); it is possible that zinc-insensitive patches may contain more glutamate receptor subunit heteromers with zinc-insensitive subunit combinations (Dreixler and Leonard 1994, 1997).

Zinc specifically augments the AMPAR currents in a concentration-dependent manner

Application of glutamate to somatic patches has been shown to activate both AMPA and NMDA receptors simultaneously (Jonas and Sakmann 1992; Spruston et al. 1995). Similar to mEPSCs, previous research has determined that the fast peak and fast decay of the glutamate-evoked current is mediated primarily by the AMPAR, whereas the NMDA receptor is the principle mediator of the slower decay component (Colquhoun et al. 1992; Spruston et al. 1995). To experimentally differentiate zinc’s effects on these receptors, antagonists were employed to isolate specific receptor subtypes prior to zinc exposure.

Since neither the kainate nor the NMDA receptor mediate the peak of the glutamate-evoked currents, we chose to initially investigate the effects of zinc on AMPAR currents. To block the NMDA receptor contribution to the glutamate-evoked currents, 30 μM d-AP5 was added to both theta glass barrels, thereby isolating the AMPAR and kainate receptor–mediated...
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To explore the concentration dependence of zinc effects on glutamate receptor responses, additional experiments were conducted using lower concentrations of zinc. With no antagonists present and 50 μM zinc in only the agonist barrel, glutamate-evoked current amplitudes were significantly augmented in five of eight patches (mean, 116.6 ± 2.8%, P < 0.05) compared with control glutamate-elicited responses (data not shown). In additional experiments using lower zinc concentrations, 30 μM D-AP5 was added to the perfusing solutions to isolate the AMPAR component of the glutamate-evoked currents. The results of these experiments are illustrated in Fig. 5. Figure 5A and B, depict the results of 1-ms pulses of 50 μM zinc coapplied with glutamate on AMPAR responses. In four of seven patches, zinc augmented AMPAR current amplitudes significantly (mean, 115.0 ± 5.4% of control glutamate-evoked currents, P < 0.05), which was not statistically different from the 50 μM zinc-induced augmentation of raw glutamate currents (P > 0.05). Zinc (50 μM) had no effects on the rise and decay kinetics of the AMPAR current decay as illustrated in Fig. 5C. When these experiments were repeated with 100 μM zinc, the AMPAR currents were significantly potentiated to 121.8 ± 2.4% in three of six patches (P < 0.05, data not shown). In addition, the AMPAR current rise and decay kinetics during 50-, 100-, and 200-μM zinc coapplication were similar, i.e., there was no significant changes in the rise and decay kinetics compared with control responses. Therefore zinc-induced augmentation of both raw glutamate and isolated AMPAR current amplitudes was concentration dependent and occurred without significant effects on response kinetics.

Inhibition of desensitization reversibly occludes zinc augmentation

Zinc’s precise mechanism of action on AMPAR currents remains unknown. A review by Smart and colleagues (Smart et

FIG. 4. Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor-mediated (AMPAR) currents are augmented by pulses of 200 μM zinc with 3 mM glutamate. A: time course of brief (1 ms) pulses of agonists demonstrate that zinc reversibly potentiated AMPAR currents. n-2-amino-5-phosphonopentanoic acid (D-AP5; 30 μM) was included in both the control and drug perfusion solutions. Hatch marks represent discontinuities in recordings as perfusion solutions were changed. B: glutamate-evoked responses (left) were augmented on coapplication with zinc (right) to 132.5 ± 9.1% of controls in 7 of 14 patches. C: normalized traces exhibit no zinc-induced alterations in rise or decay kinetics of the responses (VH = −60 mV).

FIG. 3. Zinc (200 μM) augments currents evoked by 1-ms pulses of 3 mM glutamate on perisomatic outside-out patches. A: continuous tracing depicting glutamate applications with and without zinc. Circles define the exact time points at which agonists were applied. Note that currents returned to baseline amplitudes when zinc was removed from the drug barrel. B: average of 5 traces of current responses to brief pulses of glutamate alone (far left) and glutamate with zinc (right trace) depicted on a faster time scale. Currents were augmented on average to 143.8 ± 9.8% of control glutamate responses in 5 of 8 patches by 200 μM zinc coapplication. C: traces from B are normalized, superimposed, and displayed on a faster time scale. No zinc-induced differences in the kinetics of the glutamate-evoked currents were evident (VH = −60 mV).

Zinc's precise mechanism of action on AMPAR currents does not contribute significantly to the fast peak of glutamate-evoked currents, primarily effects on the AMPAR contribution to the peak are assumed to be considered in this study. Figure 4 depicts the results of this experiment. Isolated AMPAR currents closely resembled glutamate-evoked currents and, similar to the glutamate-evoked currents, were significantly potentiated by 200 μM zinc (Fig. 4, A and B). As was the case with glutamate application, not all patches were zinc sensitive. In 7 of 14 patches, zinc significantly augmented the AMPAR current amplitude to 132.5 ± 9.1% of control AMPAR amplitudes (P < 0.05); zinc had no effect on the remaining seven patches. This augmentation level was not significantly different from zinc augmentation of glutamate currents (P > 0.05). Figure 4C depicts normalized traces of the AMPAR currents with and without zinc; similar to zinc’s effects on glutamate-evoked currents, no consistent change in rise or decay kinetics was observed with zinc coapplication. Therefore it is likely that the zinc-mediated augmentation of the glutamate-evoked responses are solely a consequence of augmented AMPAR currents. This may have physiological relevance since zinc can be released into the MF-CA3 synapses at concentrations up to 100–300 μM (Frederickson et al. 1992; Xie and Smart 1991), and excitatory neurotransmission and long-term potentiation at the MF-CA3 synapses on apical dendrites have been shown to be AMPAR and not NMDA receptor dependent (Williams and Johnston 1989; Zalutsky and Nicoll 1990).

To explore the concentration dependence of zinc effects on glutamate receptor responses, additional experiments were conducted using lower concentrations of zinc. With no antagonists present and 50 μM zinc in only the agonist barrel, glutamate-evoked current amplitudes were significantly augmented in five of eight patches (mean, 116.6 ± 2.8%, P < 0.05) compared with control glutamate-elicited responses (data not shown). In additional experiments using lower zinc concentrations, 30 μM D-AP5 was added to the perfusing solutions to isolate the AMPAR component of the glutamate-evoked currents. The results of these experiments are illustrated in Fig. 5. Figure 5, A and B, depict the results of 1-ms pulses of 50 μM zinc coapplied with glutamate on AMPAR responses. In four of seven patches, zinc augmented AMPAR current amplitudes significantly (mean, 115.0 ± 5.4% of control glutamate-evoked currents, P < 0.05), which was not statistically different from the 50 μM zinc-induced augmentation of raw glutamate currents (P > 0.05). Zinc (50 μM) had no effects on the rise and decay kinetics of the AMPAR current decay as illustrated in Fig. 5C. When these experiments were repeated with 100 μM zinc, the AMPAR currents were significantly potentiated to 121.8 ± 2.4% in three of six patches (P < 0.05, data not shown). In addition, the AMPAR current rise and decay kinetics during 50-, 100-, and 200-μM zinc coapplication were similar, i.e., there was no significant changes in the rise and decay kinetics compared with control responses. Therefore zinc-induced augmentation of both raw glutamate and isolated AMPAR current amplitudes was concentration dependent and occurred without significant effects on response kinetics.

Inhibition of desensitization reversibly occludes zinc augmentation

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Once it had been ascertained that the patch demonstrated zinc amplitude percentages, plotted against time, appear in Fig. 6 as described (see Fig. 6 legend); the normalized current am-

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rents such that they were $111.3 \pm 1.9\%$ of the rundown-subtracted current amplitudes. In addition, consistent with its mechanism of action, CTZ significantly prolonged the current decay of AMPAR currents as depicted in Fig. 6B ($316.7 \pm 57.8\%$ prolongation of decay). More importantly, CTZ oc-

cluded zinc-induced augmentation of AMPAR currents as evidenced by the reduced zinc-induced augmentation of AMPAR currents during CTZ exposure (Fig. 6, B and D). After a 10-min CTZ wash out period in normal control solution, the reversibility of CTZ’s effects was tested by reassessing AMPAR zinc sensitivity (Fig. 6C). By this time, the basal pre-CTZ AMPAR properties had been reestablished: the current deactivation rates returned to faster baseline states, and zinc once again reversibly augmented glutamate-evoked currents (Fig. 6, C and D). These data support the previously established hypothesis that reductions in desensitization may account for a significant proportion of zinc’s effects in aug-

menting peak AMPAR-mediated currents, but also suggest that additional mechanisms may also play a role, since CTZ-in-

duced occlusion of zinc’s effects was not complete (Fig. 6).

Jones and Westbrook (1995) have shown that deactivation of GABA A receptor–mediated currents is mediated by the rapid transition into and out of desensitized states. Previous reports studying glutamate receptors, however, have shown that AMPAR desensitization, following brief (1 ms) saturating pulses of glutamate, does not play a major role in shaping the rate of deactivation in CA3 patches (Colquhoun et al. 1992). Given the extremely rapid desensitization of the AMPAR, however, a portion of the AMPARs may desensitize during the 1-ms agonist applications (Colquhoun et al. 1992). Since our results using CTZ suggest that zinc may be inhibiting AMPAR desensitization, paired-pulse applications with interpulse inter-

vals ranging from 10 to 500 ms were conducted on isolated AMPARs with 3 mM glutamate alone and glutamate with 200 

mM zinc to examine zinc’s effects on desensitization using alternative methods. The first of the pair of applications was defined to occur at $t = 0$ ms. As demonstrated in Fig. 7A, AMPARs were significantly desensitized at $t = 10$ ms follow-

ing the initial glutamate application such that glutamate-evoked current amplitudes were only $47.2 \pm 3.3\%$ of the initial pulse amplitude ($n = 10$ patches), indicating that approxi-

mately $50\%$ of AMPARs were desensitized by a 1-ms appli-

cation of glutamate. With increasing interpulse intervals, how-

ever, the currents eventually recovered and returned to baseline amplitudes. The average percent recovery of the patch currents was plotted with respect to the interpulse intervals in Fig. 7C. Like results reported by previous investigators (Colquhoun et al. 1992), the curve fitted to the recovery from desensitization could be well-fitted by a single exponential curve. Zinc coap-

lication with glutamate exhibited a similar time course of recovery as illustrated in Fig. 7B ($n = 4–5$ patches). The initial pulse current amplitudes are normalized in Fig. 7, A and B, to facilitate desensitization time course recovery comparisons since zinc potentiated the AMPAR currents. Like glutamate, recovery from desensitization during zinc application with glutamate could be well-fitted by a single exponential curve, with a time constant indistinguishable from that seen in gluta-

mate alone (Fig. 7C). Since zinc appears to inhibit desensiti-

zation but does not alter the recovery from desensitization with paired applications, this suggests that zinc may specifically retard the transition of AMPARs into a desensitized state, but
has little effect on the recovery from desensitization. Zinc does not prolong the decay of AMPAR currents (Figs. 3–5), but desensitization has been demonstrated to make only a minimal contribution to deactivation of AMPARs in rapid application experiments (Colquhoun et al. 1992). It appears that zinc only inhibits desensitization as channels are opening during the rising phase of the glutamate-evoked currents.

Pulse applications of zinc have no effect on NMDA receptor–mediated currents

It is well established that tonic applications of zinc block NMDA receptor–mediated currents using both whole cell and fast application techniques (cf. Spruston et al. 1995; Traynelis et al. 1998; Westbrook and Mayer 1987). Similar to results previously reported (Spruston et al. 1995), tonically applied zinc reduced NMDA receptor–mediated currents on patches excised from CA3 neurons to 30.0 ± 4.45% (50 μM, n = 3) and 22.0 ± 9.7% (200 μM, n = 3) of control applications (data not shown). Under physiological conditions, however, subsynaptic NMDA receptors are not continuously exposed to zinc. Rather, since glutamate and zinc are colocalized within the same synaptic vesicles and co-released simultaneously, and both glutamate and zinc are rapidly taken up, NMDA receptors are only briefly exposed to zinc when glutamate is present. In contrast to zinc’s strong block of NMDA receptors when tonically applied, prior reports have demonstrated that even high concentrations of zinc (1 mM), when included only in the agonist solution, only blocks NMDA receptor–mediated currents to a very small degree and slightly slows the rise time (Spruston et al. 1995). However, in other experiments, in addition to potentiating AMPAR currents, zinc has also been shown to augment NMDA receptor–mediated currents to a very small degree and slightly slows the rise time (Spruston et al. 1995). However, in other experiments, in addition to potentiating AMPAR currents, zinc has also been shown to augment NMDA receptor–mediated currents to a very small degree and slightly slows the rise time (Spruston et al. 1995). However, in other experiments, in addition to potentiating AMPAR currents, zinc has also been shown to augment NMDA receptor–mediated currents to a very small degree and slightly slows the rise time (Spruston et al. 1995). However, in other experiments, in addition to potentiating AMPAR currents, zinc has also been shown to augment NMDA receptor–mediated currents to a very small degree and slightly slows the rise time (Spruston et al. 1995). However, in other experiments, in addition to potentiating AMPAR currents, zinc has also been shown to augment NMDA receptor–mediated currents to a very small degree and slightly slows the rise time (Spruston et al. 1995). However, in other experiments, in addition to potentiating AMPAR currents, zinc has also been shown to augment NMDA receptor–mediated currents to a very small degree and slightly slows the rise time (Spruston et al. 1995). However, in other experiments, in addition to potentiating AMPAR currents, zinc has also been shown to augment NMDA receptor–mediated currents to a very small degree and slightly slows the rise time (Spruston et al. 1995).
currents were isolated with 5 μM CNQX, and 3 mM glutamate was applied. Since the voltage-dependent magnesium block of the NMDA receptor pore is very effective at a holding potential of −60 mV, magnesium was omitted from the perfusion solutions to elicit larger, more easily analyzed currents. Even without magnesium, NMDA receptor–mediated currents were much smaller and reached peak current amplitudes much more slowly than AMPAR currents. Under these conditions, brief (1 ms) pulses of zinc (50 and 200 μM), coapplied with glutamate demonstrated no significant effect on NMDA receptor–mediated currents (P > 0.05 for both 50 and 200 μM, n = 4 for 50 μM, n = 7 for 200 μM; Fig. 8). Furthermore, zinc had no effect on the rise time or the decay time of the NMDA receptor–mediated currents. Thus pulsatile application of zinc appeared to potentiate glutamate-evoked currents by specifically enhancing AMPAR currents, without impacting NMDA receptors at all.

**DISCUSSION**

To date, there is no consensus regarding zinc’s physiological role in the CNS. One reason for this uncertainty is that zinc has an extremely wide spectrum of involvement; zinc plays a role in hundreds of processes ranging from DNA transcription to modulation of ion channels (for reviews, see Choi and Koh 1998; Smart et al. 1994). To exacerbate the confusion, these effects are often seemingly contradictory; for example, zinc has been shown to block apoptosis (Sunderman 1995), and yet it is a potent inducer of excitotoxicity (Choi and Koh 1998).
Zinc’s presence in certain specific telencephalic excitatory pathways (Perez-Clausell and Danscher 1985, 1986) and not others indicates that it most likely subserves a specific purpose at select synapses. Previous studies have demonstrated that CNS zinc concentrations of up to 300 μM are achievable on repetitive stimulation of nerve terminals (Assaf and Chung 1984; Howell et al. 1984). These concentrations are relevant because they easily exceed those necessary to impact heavily on excitatory neurotransmission (Mayer and Vyckicky 1989; Rassendren et al. 1990). Therefore since zinc is colocalized exclusively in glutamatergic pathways, modulation of excitatory neurotransmission is probably one of zinc’s chief roles. It is this potential role that is addressed in this study. Prior reports have demonstrated zinc-induced potentiation of MF-CA3 field potentials with tetanic stimulation (Budde et al. 1997) as well as zinc-induced augmentation of whole cell AMPAR currents (Mayer and Vyckicky 1989; Xie et al. 1993). In a recent study, it has been shown that, at MF-CA3 synapses, zinc tonically occupies the high-affinity binding site of NMDA receptors, and will also occupy the lower affinity binding site during action potential–dependent zinc release. Both of these effects result in zinc shaping MF synaptic responses, but do not appear to alter LTP in control animals compared with transgenic or mutant strains lacking vesicular zinc (Vogt et al. 2000). Unlike previously published results, the present study examines zinc’s effects on excitatory neurotransmission at both a single-cell and isolated receptor level in slice. By using whole cell slice patch techniques and ultrafast solution changing techniques, we demonstrate zinc-induced augmentation of mEPSCs and a concentration-dependent potentiation of AMPAR currents.

**Mechanism of zinc-induced augmentation of AMPARs**

It has been hypothesized that zinc may enhance AMPAR currents by reducing AMPAR desensitization (Bresink et al. 1996; Smart et al. 1994). In this study, evidence for such a mechanism is provided in experiments illustrated in Fig. 6, which demonstrates that CTZ, a potent AMPAR desensitization blocker, occludes zinc’s ability to augment AMPAR currents. There are three possible explanations for this loss of zinc sensitivity. First, CTZ and zinc may be competing for an allosteric modulatory site, and the tonic presence of CTZ is antagonizing zinc’s ability to bind to this site. Another possibility is that CTZ is maximally relieving AMPAR desensitization. If zinc augments AMPAR currents by inhibiting desensitization, then CTZ’s competing mechanism of action would occlude zinc’s effects. Given the vastly different molecular structures of the thiazides and zinc, it is unlikely that the two compounds would be competing for the same modulatory site. In addition, to date there have been no prior reports of zinc binding to the thiazide modulatory site. A third possibility is that CTZ may be combining with zinc and chelating it, reducing zinc effects through a receptor-independent mechanism. Although the first and third mechanisms cannot be completely ruled out, the second mechanism appears more likely, given that it is supported both by this study and by binding and whole cell electrophysiological studies (Bresink et al. 1996). Zinc may have additional mechanisms contributing to its AMPAR actions. Zinc effects on AMPAR peaks were larger than CTZ’s, and the occlusive effects of CTZ were not complete. The close apposition of zinc binding sites in the S1/S2 region of AMPARs to AMPA binding sites suggest that zinc could potentially modulate agonist binding to the receptor (Armstrong and Gouaux 2000).

**Possible mediators of zinc-sensitive and zinc-insensitive AMPA responses**

One consistent finding throughout all of the above-described patch experiments is that only 60% of the patches were zinc sensitive. This was not true for AMPAR-mediated synaptic responses, where the majority of cells (6/7) had mEPSCs that were augmented by zinc (Figs. 1 and 2). One potential factor that could mediate this bimodal responsiveness of AMPARs in patches could be differences in the subunit composition of extrasynaptic, but not synaptic, receptors. A recently published study has characterized five putative zinc-binding histidine residues in the S1/S2 region of AMPARs and found that some of these histidines are not conserved in all subunits, in particular His-412, which is present in GluR2–4, but absent in GluR1 (Armstrong and Gouaux 2000). This agrees well with Xenopus oocyte data that demonstrated that AMPARs composed of homomeric GluR3 subunits were zinc sensitive, while homomeric GluR1 AMPARs were not (Drexler and Leonard 1994, 1997). There may be additional complexity in AMPAR subunit determinants of zinc sensitivity: flip AMPAR splice variants may be more zinc sensitive than flop variants (Shen and Yang 1999). These data, combined with our patch data, suggest that, in CA3 neurons, zinc-sensitive synaptic AMPARs may be primarily comprised of GluR2–4 subunits and/or flip splice variants, while extrasynaptic AMPARs may have a higher contribution of GluR1-containing receptors, and/or a higher contribution of flop splice variants, conferring zinc insensitivity to 40–50% of patches.

Data from in situ hybridization, immunohistochemical, and single cell expression profiling studies have provided extensive information concerning the AMPAR subunits expressed by 15- to 20-day-old rat CA3 neurons. Using mRNA expression profiles in individual CA3 pyramidal neurons from 12- to 17-day-old rats, Geiger et al. (1995) demonstrated that these neurons expressed mostly GluR1 and GluR2, lower levels of GluR3, and no GluR4 mRNA, which agrees relatively well with in situ hybridization studies (Keinänen et al. 1990; Pellegrini-Giampietro et al. 1991). Individual CA3 neurons also expressed primarily flip splice variants, with lower levels of expression of flop splice variants (Geiger et al. 1995). This is also in relatively good agreement with in situ hybridization studies, which demonstrated that GluR1 and 2 flip mRNAs are present early in development, while flop mRNAs gradually increase expression over the first 2–3 postnatal weeks (Monyer et al. 1991). Given that flop splice variants may be differentially insensitive to zinc modulation (Shen and Yang 1999), this developmental enhancement in flop expression in 2- to 3-wk-old animals might contribute to zinc insensitivity in some patches pulled from 2- to 3-wk-old CA3 neurons.

Immunohistochemical studies in adult rat CA3 region have demonstrated that GluR1 staining is associated with pyramidal neurons and is particularly strong in CA3 dendritic regions, while immunostaining for GluR 2/3 is primarily localized in the somatic layer, with weaker (but still noticeable) staining in dendritic regions (Leranth et al. 1996). This suggests that both GluR1 and GluR2/3 subunits may combine to form AMPARs...
in perisomatic membranes, which in turn would constitute the receptors studied in pulled patch experiments. Immunogold studies in area CA3 of adult rats examining subcellular distribution of AMPAR subunits demonstrated strong localization of GluR2/3 and 4 subunits and weaker localization of GluR1 subunits at mossy fiber synapses, suggesting that synaptically released glutamate may preferentially activate GluR2−4 subunit-enriched AMPARs (Baude et al. 1995). In contrast, cell bodies in region CA3 labeled for both GluR1 and GluR2/3 subunits (Baude et al. 1995; Leranth et al. 1996). Given that GluR1-containing AMPARs may be relatively zinc insensitive (Dreixler and Leonard 1994, 1997), this suggests that AMPARs in perisomatic patches may have a higher proportional expression of GluR1 subunits than AMPARs activated during mEPSCs. This in turn could contribute to the finding that 40% of perisomatic AMPAR responses were zinc insensitive, while only 14% of CA3 neurons had zinc-insensitive synaptic responses.

Possible roles of zinc in the CNS

Although spontaneous zinc release has been reported (Charlton et al. 1985), repetitive activation of excitatory pathways appears to be the principle stimulus for zinc release in the CNS (Assaf and Chung 1984; Budde et al. 1997; Howell et al. 1984). Given this finding and the fact that tonically applied zinc inhibits NMDA receptor-mediated currents, some investigators initially proposed that zinc may serve to inhibit excitotoxicity (Kida and Matyja 1990; Peters et al. 1987; Weiss et al. 1993). The present study, as well as others (Spruston et al. 1995), has shown that, rather than inhibiting neuronal excitability, zinc enhances neuronal excitability (Mayer and Vytklisky 1989) and strongly potentiates excitotoxicity (for review, see Choi and Koh 1998). Although the precise mechanisms by which zinc mediates excitotoxicity remains unknown, previous studies have demonstrated that zinc has numerous effects on ion channels and neurotransmitter release that tend to potentiate excitability (Choi and Koh 1998). For instance, at concentrations of 300 μM, it is possible that zinc diffuses from glutamatergic synapses to sites containing zinc-sensitive GABA<sub>A</sub> receptors, thereby decreasing regional inhibitory tone (Buhl et al. 1996; Gibbs et al. 1997; Shumate et al. 1998). In addition, zinc suppresses glutamate uptake by inhibiting the glutamate transporter EAAT1 (Spirdon et al. 1998; Vandenberg et al. 1998), which may lead to higher basal levels of glutamate at excitatory nerve terminals. Thus under physiological release conditions, zinc’s depression of GABA<sub>A</sub> receptor function, coupled with an augmentation of AMPAR currents and decreased glutamate uptake, overall may predispose the limbic system to enhanced excitatory neurotransmission.

A zinc-mediated increase in MF-CA3 synaptic strength may serve to facilitate LTP induction. Given that zinc may act similarly to the thiaziode family of AMPAR modulators (Fig. 6), this could enhance synaptic plasticity. Thiazides have been shown to facilitate LTP induction in other hippocampal regions (as reviewed in Yamada 1998), most likely by augmenting EPSC amplitudes and prolonging EPSC decays. Since zinc appears to have a mechanism of action similar to these compounds, zinc could potentially serve as an endogenous AMPAR modulator (Harrison and Gibbons 1994; Huang 1997) capable of lowering the stimulus threshold required for LTP induction (however, see Vogt et al. 2000; Xie and Smart 1994).

This study was undertaken to determine zinc’s effects in situ on native receptors that normally receive zinc-containing synaptic innervation. The results demonstrate that zinc augments glutamate-evoked currents in both synaptic and extra-synaptic receptors in a concentration-dependent manner. Preliminary investigation of zinc’s mechanism of action suggests that a relief of AMPAR desensitization may contribute substantially to its modulatory actions (Bresink et al. 1996). The potentiation of AMPAR currents, coupled with reports that zinc can affect protein kinase C (PKC) (Baba et al. 1991), a protein known to play a role in the maintenance phase of LTP (Kuba and Kumamoto 1990; Linden and Routtenberg 1989), suggest that the hypothesis that one of zinc’s physiological roles may be to modulate function of glutamatergic synapses. Because LTP may be a synaptic modulatory mechanism important in learning and memory (Bliss and Collingridge 1993), zinc may play a pivotal role in these higher cognitive functions as well. The studies of Vogt et al. (2000) using ZnT-3 knockout animals lacking vesicular zinc argue against a role for zinc modulation of LTP. However, recent reports using the same ZnT-3 knockout transgenic strain have suggested that not all physiologically relevant zinc may be localized within ZnT3-decorated vesicles, since seizure-induced zinc-mediated excitotoxic mechanisms in hippocampus persist unabated in animals lacking vesicular zinc (Lee et al. 2000). In addition to a physiological role, zinc may be a major factor in certain pathological states such as epilepsy, in which zinc is aberrantly released from sprouted mossy fibers (Babb et al. 1991; Houser et al. 1990; Sutula et al. 1989; Tauck and Nadler 1985). In this environment, zinc’s inhibition of GABA<sub>A</sub> receptors, coupled with the zinc-induced augmentation of AMPAR currents, may have catastrophic ramifications on limbic excitability (Brooks-Kayal et al. 1998; Buhl et al. 1996; Gibbs et al. 1997; Shumate et al. 1998). Further research is required for a more definitive characterization of zinc’s possible roles in these pathological conditions.

We thank Drs. Annette M. L. McClellan and Stefano Vicini for generous time and assistance in assembling the solution-exchange system.

This research was supported by National Institute of Neurological Disorders and Stroke Grant RO1-NS-32403 to D. A. Coulter and by the Sophie and Nathan Gumenick Neuroscience and Alzheimer’s Research Fund. D. D. Lin was supported by the MD/PhD program of the Medical College of Virginia, Virginia Commonwealth University.

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