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Effects of Zinc on Spontaneous Miniature GABA Release in Rat Hippocampal CA3 Pyramidal Neurons

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The effects of Zn^{2+} on spontaneous glutamate and GABA release were tested in mechanically dissociated rat CA3 pyramidal neurons which retained functional presynaptic nerve terminals. The spontaneous miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs, respectively) were pharmacologically isolated and recorded using whole-cell patch clamp technique under voltage-clamp conditions. Zn^{2+} at a lower concentration (30 μM) increased GABAergic mIPSC frequency without affecting mIPSC amplitude, but it decreased both mIPSC frequency and amplitude at higher concentrations ($\geq$300 μM). In contrast, Zn^{2+} (3 to 100 μM) did not affect glutamatergic mEPSCs, although it slightly decreased both mIPSC frequency and amplitude at 300 μM concentration. Facilitatory effect of Zn^{2+} on GABAergic mIPSC frequency was occluded either in Ca^{2+}-free external solution or in the presence of 100 μM 4-aminopyridine, a non-selective K^+ channel blocker. The results suggest that Zn^{2+} at lower concentrations depolarizes GABAergic nerve terminals by blocking K^+ channels and increases the probability of spontaneous GABA release. This Zn^{2+}-mediated modulation of spontaneous GABAergic transmission is likely to play an important role in the regulation of neuronal excitability within the hippocampal CA3 area.

Key Words: Zinc, mEPSCs, mIPSCs, GABAergic terminals, Hippocampus

INTRODUCTION

The hippocampus is one of the most important areas to process higher brain functions. In the hippocampus, mossy fibers which arise from dentate gyrus granule cells and making powerful excitatory synases onto CA3 pyramidal neurons are of great interest, because of their distinct anatomical and physiological properties (Chicurel & Harris, 1992; Acsady et al, 1998; Henze et al, 2000). Short- and long-term changes in the synaptic plasticity at these synapses are also closely related to the normal physiological functions such as learning and memory (Xie & Smart, 1994; Lu et al, 2000). In general, mossy fibers release glutamate as a major neurotransmitter, however they also release Zn^{2+} and even GABA (Assaf & Chung, 1984; Walker et al, 2001). Endogenous Zn^{2+} released from mossy fiber terminals affects many important physiological consequences such as the regulation of the excitability of CA3 pyramidal neurons by modulating ionotropic glutamate receptors including AMPA and NMDA receptors, $GABA_A$ receptors and other ion channels (Westbrook & Mayer, 1987; Draguhn et al, 1990; Harrison & Gibbons, 1994; Miralles et al, 1994; Smart et al, 1994; Easaw et al, 1999; Kerchner et al, 2000). Therefore, endogenous Zn^{2+} has been suggested to play a pivotal role in the maintenance of higher brain functions in the hippocampus. While the specific actions of Zn^{2+} on receptors or ion channels have been well documented, much less is known about the functional roles of Zn^{2+} in neurotransmitter release. Although Zn^{2+} modulates presynaptic ATP-sensitive K^+ (K_{ATP}) channels to decrease action potential-dependent glutamate release from mossy fiber terminals (Bancila et al, 2004; Quinta-Ferreira & Matias, 2005), it is still unknown whether Zn^{2+} can regulate GABA release. In the present study, therefore, we have investigated whether Zn^{2+} can modulate spontaneous GABA release from presynaptic terminals which innervate onto CA3 pyramidal neurons.

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METHODS

Preparation

All experiments were carried out according to the guiding principles for the care and use of animals approved by the Council of the Physiological Society of Korea and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and every effort was made to minimize both the number of animals used and their suffering.

ABBREVIATIONS: mEPSCs, spontaneous miniature excitatory postsynaptic currents; mIPSCs, spontaneous miniature inhibitory postsynaptic currents; SR95531, 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; APV, DL-2-amino-5-phosphonovaleric acid; 4-AP, 4-aminopyridine; TTX, tetrodotoxin; VDCCs, voltage-dependent Ca^{2+} channels.

Wistar rats (12~15 d old) were decapitated under pentobarbital anesthesia (50 mg/kg, i. p.). The brain was dissected and transversely sliced at a thickness of 370 μm using a microslicer (VT1000S; Leica, Nussloch, Germany). Slices containing the hippocampus were kept in an incubation medium (see below) saturated with 95% O_2 and 5% CO_2 at room temperature (22~24°C) for at least 1 h before mechanical dissociation. For dissociation, slices were transferred on a 35 mm culture dish (Primaria 3801; Becton Dickinson, Rutherford, NJ) containing a standard external solution (see below), and the hippocampal region was identified under a binocular microscope (SMZ-1; Nikon, Tokyo, Japan). Details of the mechanical dissociation have been described previously (Rhee et al, 1999; Jang et al, 2005). Briefly, mechanical dissociation was accomplished using a custom-built vibration device and a fire-polished glass pipette oscillating at about 50~60 Hz (0.3~0.5 mm) on the surface of the hippocampal region. Slices were removed, and the mechanically dissociated neurons were allowed to settle and adhere to the bottom of the dish for 15 min. These dissociated neurons loose the most distal processes, but retain a short portion (~100 μm in a length) of their thick proximal dendrites.

Electrical measurements

All electrical measurements were performed using conventional whole-cell patch recordings and a standard patch-clamp amplifier (Axopatch 200B; Axon Instruments; Union City, CA, USA), except where indicated. Neurons were voltage clamped at a holding potential (V_H) of −60 mV, except where indicated. Patch pipettes were made from borosilicate capillary glass (1.5 mm outer diameter, 0.9 mm inner diameter; G-1.5; Narishige, Tokyo, Japan) in two stages on a pipette puller (P-97; Shutter Instrument Co.). The resistance of the recording pipettes filled with internal solution was 4~6 MΩ. The liquid junction potential and pipette capacitance were compensated. Neurons were viewed under phase contrast on an inverted microscope (TE-2000; Nikon, Tokyo, Japan). Membrane currents were filtered at 1 kHz (E-3201A Decade Filter; NF Electronic Instruments, Tokyo, Japan), digitized at 4 kHz, and stored on a computer equipped with pCLAMP 8.02 (Axon Instruments). When recording, 10 mV hyperpolarizing step pulses (30 ms in duration) were periodically delivered to monitor the access resistance. All experiments were performed at room temperature (22~24°C).

Data analysis

Spontaneous mEPSCs and mIPSCs were counted and analyzed using the MiniAnalysis program (Synaptosoft Inc., Decatur, GA) as described previously (Jang et al, 2002; 2006). Briefly, mEPSCs and mIPSCs were screened automatically using an amplitude threshold of 10 pA and then visually accepted or rejected based upon the rise and decay times. The average values of both the frequency and amplitude of spontaneous events during the control period (5~10 min) were calculated for each recording, and the frequency and amplitude of all the events during different experimental conditions were normalized to these values. The effects of Zn^{2+} were quantified as a percentage increase in either mEPSC or mIPSC frequency, compared to the control values. Numerical values are presented as mean ±standard error of the mean (SEM) using values normalized to the control, except where indicated. Significant differences in the mean amplitude and frequency were tested using Student's paired two-tailed *t*-test and using absolute values rather than normalized ones. Values of $p < 0.05$ were considered significant.

Solutions

The ionic composition of the incubation medium consisted of (in mM) 124 NaCl, 3 KCl, 1.5 KH_2PO_4, 24 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgSO_4$ and 10 glucose saturated with 95% O_2 and 5% CO_2. The pH was about 7.45. The standard external solution consisted of (in mM) 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Hepes, and pH was adjusted to 7.4 with Tris-base. The Ca^{2+}-free external solution consisted of (in mM) 150 NaCl, 5 KCl, 3 $MgCl_2$, 10 glucose and 10 Hepes, and pH was adjusted to 7.4 with Tris-base. For recording spontaneous synaptic currents, the standard external solutions routinely contained 500 nM tetrodotoxin (TTX) and 50 μM DL-2-amino-5-phosphonovaleric acid (APV) to block voltage-dependent Na^+ channels and NMDA receptors, respectively. The ionic composition of the internal (pipette) solution consisted of (in mM) 140 Cs-methanesulfonate, 5 TEA-Cl, 5 CsCl, 2 EGTA and 10 Hepes with

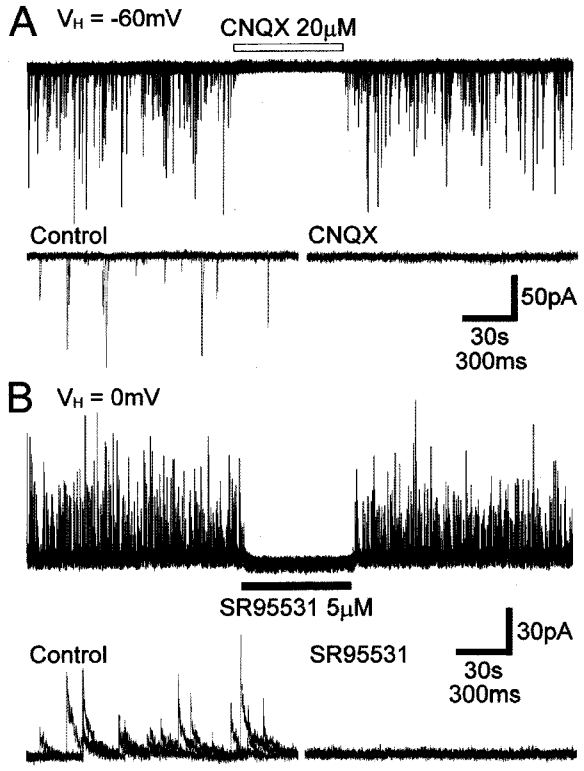

Fig. 1. Isolation of mEPSCs and mIPSCs from mechanically dissociated CA3 pyramidal neurons. (A) A typical trace of glutamatergic mEPSCs recorded before, during and after the application of 20 μM CNQX at a V_H of −60 mV. The external solution contained 500 nM TTX, 50 μM APV and 5 μM SR95531. Insets represent typical traces with an expanded time scale. (B) A typical trace of GABAergic mIPSCs recorded before, during and after the application of 5 μM SR95531 at a V_H of 0 mV. The external solution contained 500 nM TTX, 50 μM APV and 20 μM CNQX. Insets represent typical traces with an expanded time scale.

a pH adjusted to 7.2 with Tris-base.

Drugs

The drugs used in the present study were $ZnCl_2$, TTX, APV, 4-aminopyridine (4-AP) 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid (SR95531), 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX), EGTA, ATP-Mg (from Sigma, St. Louis, MO). All solutions containing drugs were applied using the 'Y-tube system' for rapid solution exchange.

RESULTS

Pharmacological isolation of mEPSCs and mIPSCs

The mEPSCs were recorded at a holding potential (V_H) of −60 mV in the presence of 500 nM TTX, 50 μM APV and 5 μM SR95531, which block voltage-dependent Na^+ channels, NMDA and $GABA_A$ receptors, respectively. They were reversibly blocked by 20 μM CNQX, suggesting that they are AMPA/KA receptor-mediated mEPSCs (Fig. 1A). The mIPSCs were recorded at a V_H of 0 mV in the presence of 500 nM TTX, 50 μM APV and 20 μM CNQX, and were reversibly eliminated by 5 μM SR95531, suggesting that they are $GABA_A$ receptor-mediated mIPSCs (Fig. 1B).

Effects of Zn^{2+} on mEPSCs and mIPSCs

First, the effect of Zn^{2+} on spontaneous GABA release was tested as shown in Fig. 2A and B. 30 μM Zn^{2+} increased mIPSC frequency to 158.6±12.2% (n=8, $p<0.05$) without affecting the current amplitude (95.5±8.4%, n=8, p=0.42) (Fig. 2A, B). Fig. 2B shows that the distribution of mIPSC frequency was shifted to the left by Zn^{2+} ($p<0.01$, Kolmogorov-Smirnov [K-S] test), but that of mIPSC amplitude was not changed (p=0.11, K-S test), suggesting that Zn^{2+} increases the probability of spontaneous GABA release by presynaptic but not postsynaptic mechanism. However, 300 μM Zn^{2+} greatly decreased both mIPSC frequency (62.3±6.7% of the control, n=6, $p<0.05$) and amplitude (34.6±4.5% of the control, n=6, $p<0.01$) (Fig. 2C, 2D). As shown in Fig. 2D, Zn^{2+} at lower concentrations (10 to 30 μM) increased mIPSC frequency, but it decreased both mIPSC frequency and amplitude at higher concentrations (≥300 μM).

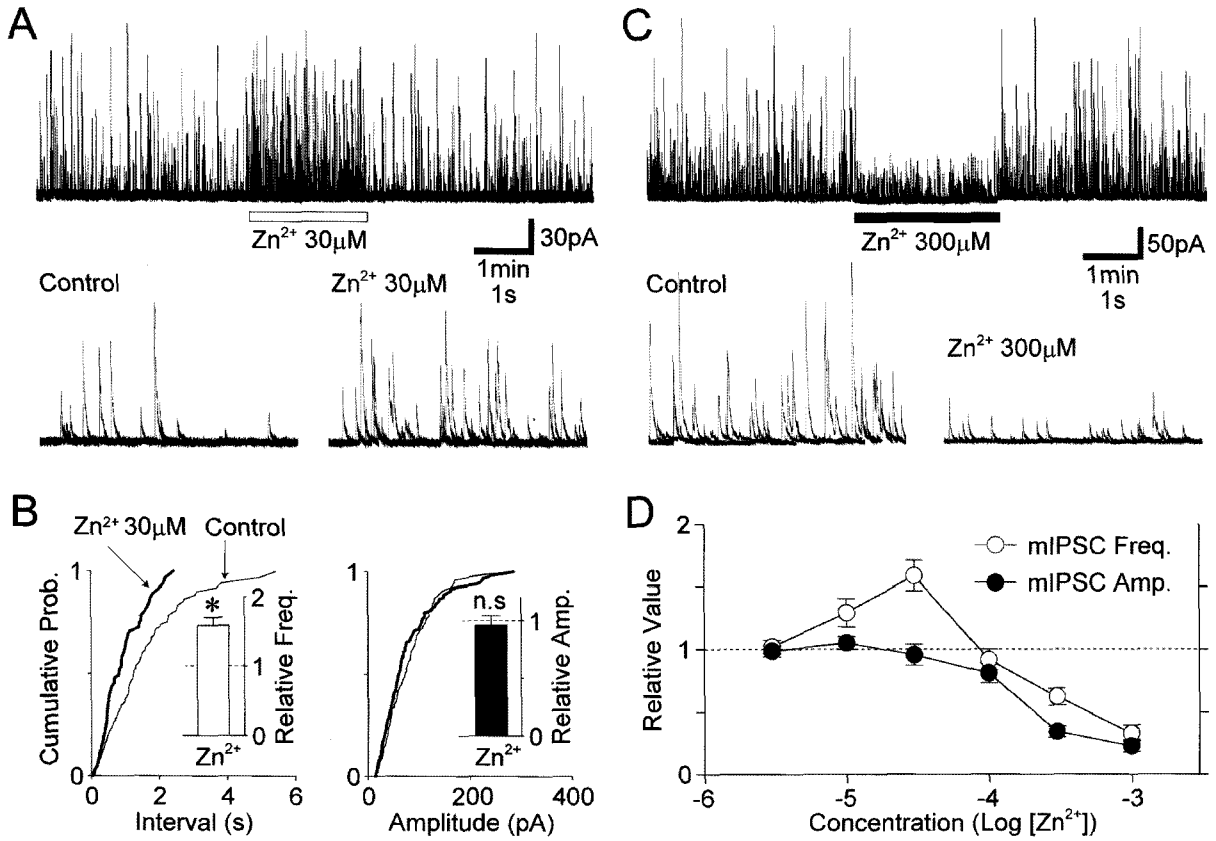

Fig. 2. Effect of Zn^{2+} on GABAergic mIPSCs. (A) A typical trace of GABAergic mIPSCs recorded before, during and after the application of 30 μM Zn^{2+}. Insets represent typical traces with an expanded time scale. (B) Cumulative probability distributions for inter-event interval (left) and current amplitude (right) of GABAergic mIPSCs, shown in A. 197 events for control and 218 events for Zn^{2+} were plotted. Insets, Zn^{2+}-induced changes in mIPSC frequency (left) and amplitude (right). Each column represents mean and SEM from 8 experiments. *: $p<0.05$, n.s: not significant. (C) A typical trace of GABAergic mIPSCs recorded before, during and after the application of 300 μM Zn^{2+}. Insets represent typical traces with an expanded time scale. Note that 300 μM Zn^{2+} greatly reduced mIPSC amplitude. (D) Concentration-response relationship for GABAergic mIPSC frequency (open circles) and amplitude (closed circles). All points represent mean and SEM from 6 to 8 experiments.

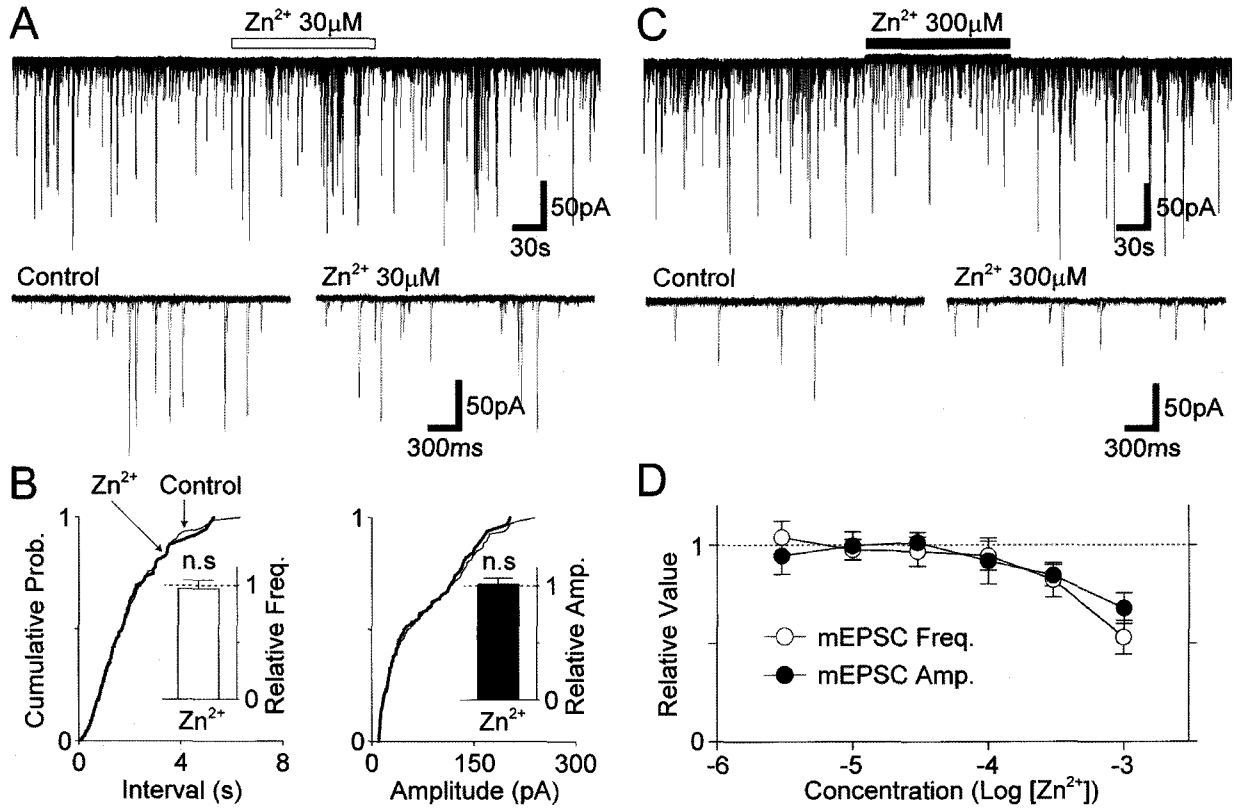

Fig. 3. Effect of Zn^{2+} on glutamatergic mEPSCs. (A) A typical trace of glutamatergic mEPSCs recorded before, during and after the application of 30 μM Zn^{2+}. Insets represent typical traces with an expanded time scale. (B) Cumulative probability distributions for inter-event interval (left) and current amplitude (right) of mEPSCs shown in (A) 182 events for control and 126 events for Zn^{2+} were plotted. Insets, Zn^{2+}-induced changes in mIPSC frequency (left) and amplitude (right). Each column represents mean and SEM from 7 experiments. n.s: not significant. (C) A typical trace of glutamatergic mEPSCs recorded before, during and after the application of 300 μM Zn^{2+}. Insets represent typical traces with an expanded time scale. (D) Concentration-response relationship for glutamatergic mEPSCs frequency (open circles) and amplitude (closed circles). All points represent mean and SEM from 5 to 7 experiments.

We also tested whether Zn^{2+} can modulate spontaneous glutamate release, and found that 30 μM Zn^{2+} affected neither mEPSC frequency (96.8±5.3%, n=6, p=0.35) nor current amplitude (101.3±5.4%, n=6, p=0.83) (Fig. 3A, 3B). As shown in Fig. 3B, 30 μM Zn^{2+} did not affect the distributions of mEPSC frequency (p=0.93, K-S test) or amplitude (p=0.78, K-S test). However, 300 μM Zn^{2+} slightly decreased both mEPSC frequency (82.3±8.8% of the control, n=6, $p<0.05$) and amplitude (84.5±5.6% of the control, n=6, $p<0.05$) (Fig. 3C, 3D). As evident in concentration-response relationship, Zn^{2+} at lower concentrations ($\leq$100 μM) did not affect mEPSC frequency and amplitude, but it decreased both mEPSC frequency and amplitude at higher concentrations ($\geq$300 μM) (Fig. 3D).

Zn^{2+} depolarizes GABAergic presynaptic terminals

To investigate mechanisms underlying the Zn^{2+}-induced increase of mIPSC frequency, we studied the effect of Ca^{2+}-free external solution. Exposure of CA3 pyramidal neurons to Ca^{2+}-free external solution decreased GABAergic mIPSC frequency to 60.8±6.3% of the control (n=7, $p<0.01$) with little changes in mIPSC amplitude (92.5±7.7% of the control, p=0.20, n=7) (Fig. 4). The above results indicate that the occurrence of GABAergic spontaneous mIPSCs might be partially dependent on extracellular Ca^{2+}, and that mIPSCs observed in the Ca^{2+}-free external solution might be classical minis, which is not dependent on Ca^{2+} influx from extracellular space. In this condition, 30 μM Zn^{2+} failed to increase mIPSC frequency (93.4±9.7% of the Ca^{2+}- free condition, n=7, p=0.62) (Fig. 4A and B). We also examined the effect of 4-AP, a general K^+ channel blocker (Harvey & Marshall, 1977), on the Zn^{2+}-induced increase of mIPSC frequency. Application of 100 μM 4-AP greatly increased GABAergic mIPSC frequency to 629.9±103.6% of the control (n=6, $p<0.05$) with little changes in mIPSC amplitude (108.5±10.4% of the control, p=0.39, n=6) (Fig. 4). The results suggest that the blockade of presynaptic K^+ channels depolarizes GABAergic nerve terminals, and that resultant presynaptic depolarization activates VDCCs, and thus increasing GABAergic mIPSC frequency. In the presence of 4-AP, however, 10 μM Zn^{2+} again failed to affect mIPSC frequency (90.7±14.1% of the 4-AP condition, n=6, p=0.28) (Fig. 4).

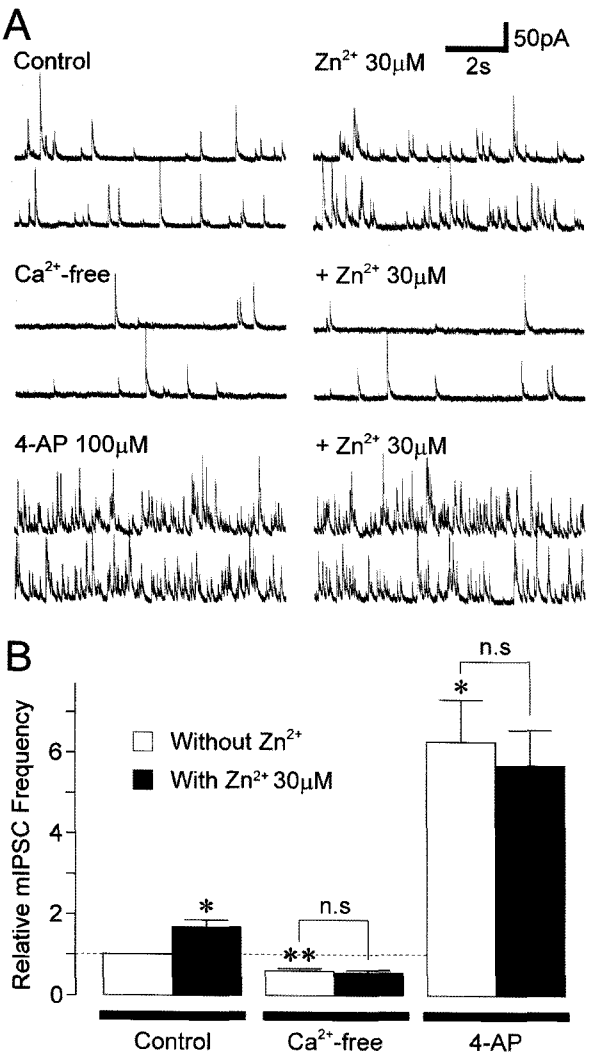

Fig. 4. Effects of Ca^{2+}-free external solution and 4-AP on Zn^{2+}-mediated increase in GABAergic mIPSC frequency. (A) Typical traces of GABAergic mIPSCs recorded before (left) and during (right) the application of 30 μM Zn^{2+} in the control external solution (upper), in the Ca^{2+}-free external solution (middle) and in the presence of 100 μM 4-AP (lower). All traces were recorded from the same neuron. (B) Zn^{2+}-induced changes in GABAergic mIPSC frequency either in the Ca^{2+}-free external solution (n=7) or in the presence of 100 μM 4-AP (n=6). All columns were normalized to the control frequency. *: $p<0.05$, **: $p<0.01$, n.s: not significant.

DISCUSSION

It has been well known that physiological roles of endogenous Zn^{2+} are mediated by the modulation of a variety of receptors and ion channels (Westbrook & Mayer, 1987; Draguhn et al, 1990; Harrison & Gibbons, 1994; Miralles et al, 1994; Smart et al, 1994; Easaw et al, 1999; Kerchner et al, 2000). Although Zn^{2+} affects primarily the activities of such receptors and/or ion channels on postsynaptic membrane, it regulates also neurotransmitter release by affecting resting membrane potential or presynaptic Ca^{2+} entry of nerve terminals. For example, Zn^{2+} inhibits depolarization-induced glutamate release via activation of K_{ATP} channels and/or inhibition of voltage-dependent Ca^{2+} channels (VDCCs) on mossy fiber terminals (Bancila et al, 2004; Quinta-Ferreira & Matias, 2005). However, Zn^{2+} seems to have differential effects on GABAergic transmission, where it enhances GABA release from presynaptic terminals innervating onto the dendritic region of CA3 pyramidal neurons, but inhibits GABA release onto pyramidal neuron soma (Ricciardi & Malouf, 1995). Detailed mechanism remains poorly understood.

In the present study, we found that low concentrations of Zn^{2+} (30 μM) increased GABAergic mIPSC frequency without affecting the current amplitude, indicating that Zn^{2+} acts presynaptically to increase spontaneous GABA release. The Zn^{2+}-induced increase in mIPSC frequency was completely occluded in the Ca^{2+}-free external solution, suggesting that the facilitatory action of Zn^{2+} requires Ca^{2+} influx from the extracellular space. In addition, the Zn^{2+}-induced increase of mIPSC frequency was attenuated, when K^+ channels were blocked by 4-AP, a general K^+ channel blocker. Therefore, Zn^{2+} is likely to depolarize GABAergic presynaptic nerve terminals by inhibiting presynaptic K^+ channels such as delayed rectifier K^+ channels or Ca^{2+}-activated K^+ channels (Miralles et al, 1994) and, subsequently, the presynaptic depolarization activates VDCCs and increases spontaneous GABA release. However, Zn^{2+} at higher concentrations ($\geq$300 μM) decreased both the frequency and amplitude of GABAergic mIPSCs. These results might be due to Zn^{2+}-mediated direct inhibition of presynaptic VDCCs, as Zn^{2+} at higher concentrations ($\geq$ 100 μM) fully blocks VDCCs (Kerchner et al, 2000). As a result, the inhibition of presynaptic VDCCs would reduce the release probability by inhibiting presynaptic Ca^{2+} entry. The inhibition of postsynaptic $GABA_A$ receptors by Zn^{2+} would also contribute to a decrease of mIPSC amplitude, leading to a reduction in the number of detected mIPSCs. These results together suggest that Zn^{2+} differentially modulates spontaneous GABA release by affecting the activities of presynaptic K^+ and Ca^{2+} channels in a concentration-dependent manner.

On the other hand, Zn^{2+} ($\leq$100 μM) had no effect on glutamatergic mEPSCs, although it significantly decreased both the frequency and amplitude of mEPSCs at higher concentrations ($\geq$300 μM). One explanation on the differential effects of Zn^{2+} on mIPSCs and mEPSCs might be that Zn^{2+}-sensitive K^+ channels expressed on GABAergic terminals might not exist in glutamatergic nerve terminals. Alternatively, hyperpolarization of presynaptic membrane due to an increase in K_{ATP} and/or A-type K^+ currents by Zn^{2+} (Huang et al, 1993; Bancila et al, 2004) might reduce the degree of Zn^{2+}-induced presynaptic depolarization. However, further study would be needed to reveal whether the expression patterns of specific K^+ channels differ in excitatory and inhibitory nerve terminals.

The extracellular concentration of Zn^{2+} reached in the CA3 area after synaptic release is still a matter of debate. The estimated Zn^{2+} concentrations after high frequency stimulation range between 10 and 100 μM (Vogt et al, 2000; Li et al, 2001), even up to 300 μM during intense neural activity or epileptiform discharges (Assaf & Chung, 1984). Zn^{2+} at concentrations as high as 100 μM can lead to cytotoxic effects, resulting from Zn^{2+} entry into neurons via Zn^{2+}-permeable ion channels (e.g., AMPA, NMDA receptors and VDCCs), with ultimate consequence of disruption of intracellular enzymatic functions (Frederickson & Moncrieff, 1994; Frederickson et al, 2004). On the other hand, Zn^{2+} at lower concentrations seems to be neuroprotective

against ischemic damage (Bancila et al, 2004), as Zn^{2+} chelation or Zn^{2+} deficiency is associated with increased seizure susceptibility (Noebels & Sidman, 1989; Mitchell & Barnes, 1993). In this regard, the present results would provide additional evidence that Zn^{2+} at low concentrations might be neuroprotective against excessive glutamate excitotoxicity by increasing the inhibitory tone (GABAergic but not glutamatergic transmission). Nevertheless, the extracellular concentration of Zn^{2+}, which reaches around GABAergic presynaptic nerve terminals, should be determined.

In conclusion, our present results suggest that Zn^{2+} regulates differentially the probability of spontaneous GABA release in a concentration-dependent manner. This Zn^{2+}-mediated differential modulation of spontaneous GABAergic transmission is likely to play an important role in the maintenance of higher brain functions in the hippocampus as well as in the regulation of excitability of CA3 pyramidal neurons within the hippocampal CA3 area.

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