

Endogenous Zinc Inhibits GABA_A Receptors in a Hippocampal Pathway

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Ruiz, Arnaud, Matthew C. Walker, Ruth Fabian-Fine, and Dimitri M. Kullmann. Endogenous zinc inhibits GABA_A receptors in a hippocampal pathway. *J Neurophysiol* 91: 1091–1096, 2004. First published October 15, 2003; 10.1152/jn.00755.2003. Depending on their subunit composition, GABA_A receptors can be highly sensitive to Zn²⁺. Although a pathological role for Zn²⁺-mediated inhibition of GABA_A receptors has been postulated, no direct evidence exists that endogenous Zn²⁺ can modulate GABAergic signaling in the brain. A possible explanation is that Zn²⁺ is mainly localized to a subset of glutamatergic synapses. Hippocampal mossy fibers are unusual in that they are glutamatergic but have also been reported to contain GABA and Zn²⁺. Here, we show, using combined Timm's method and post-embedding immunogold, that the same mossy fiber varicosities can contain both GABA and Zn²⁺. Chelating Zn²⁺ with either calcium-saturated EDTA or *N,N,N',N'*-tetrakis (2-pyridylmethyl)ethylenediamine had no effect on stratum-radiatum-evoked inhibitory postsynaptic currents (IPSCs), but enhanced IPSCs evoked by stimuli designed to recruit dentate granule cells. We also show that IPSCs recorded in CA3 pyramidal neurons in acute hippocampal slices are depressed by exogenous Zn²⁺. This depression was of similar amplitude whether the IPSCs were evoked by stimulation in s. radiatum (to recruit local interneurons) or in the s. granulosum of the dentate gyrus (to recruit mossy fibers). These results show for the first time that GABAergic IPSCs can be modulated by endogenous Zn²⁺ and are consistent with GABA release at Zn²⁺-containing mossy fiber synapses.

INTRODUCTION

Zinc is widely distributed in the mammalian brain. Ionic Zn²⁺ is concentrated in glutamatergic synaptic varicosities and is especially abundant in hippocampal mossy fibers, which project from dentate granule cells to the hilus and CA3. It is present in presynaptic vesicles and is released in response to depolarization (Assaf and Chung 1984; Howell et al. 1984; reviewed by Frederickson et al. 2000). Exogenously applied Zn²⁺ powerfully inhibits GABA_A and *N*-methyl-D-aspartate (NMDA) receptors (Draguhn et al. 1990; Smart et al. 1994; Westbrook and Mayer, 1987) and glutamate transporters (Spiridon et al. 1998; Vandenberg et al. 1998). Zn²⁺ also has biphasic effects on glycine receptors (Bloomenthal et al. 1994). At higher concentrations, Zn²⁺ also acts on several ion channels (Busselberg et al. 1992; Winegar and Lansman, 1990). Despite this wealth of evidence, the role of endogenous Zn²⁺ in modulating these processes has remained elusive.

Recent physiological evidence shows that endogenous Zn²⁺ can modulate excitatory synaptic transmission (Molnar and Nadler, 2001b; Ueno et al. 2002; Vogt et al. 2000). GABA_A receptors are also very sensitive to Zn²⁺ (Westbrook and Mayer, 1987) and have a discrete Zn²⁺ binding site that differs

among GABA_A receptor subunits (Hosie et al., 2003). As yet, there has been no demonstration that endogenous Zn²⁺ can modulate GABA_A receptors. Indeed, stimulation of mossy fibers under conditions where Zn²⁺-mediated modulation of NMDA receptors can be demonstrated did not affect GABA receptor-mediated currents evoked in granule cells by photo-uncaging GABA (Molnar and Nadler, 2001a). This contrasted with the finding that exogenous Zn²⁺ (200 μM) application attenuated the GABA_A receptor-mediated currents. Among possible explanations for this negative result is that synaptically released Zn²⁺ is confined to excitatory synapses and that Zn²⁺ spillover to GABAergic synapses is not sufficient to modulate the GABA_A receptors present. Indeed, the typical distance and diffusional barriers separating Zn²⁺ release sites from GABA_A receptors are likely to be greater than those separating Zn²⁺ release sites from NMDA receptors at most glutamatergic synapses. A possible exception is at mossy fiber synapses on CA3 pyramidal neurons: these contain relatively high concentrations of GABA (Sandler and Smith, 1991; Sloviter et al. 1996). Some recent reports have shown that stimuli designed to activate mossy fibers can give rise to inhibitory postsynaptic potentials and currents (IPSPs and IPSCs) in CA3 pyramidal cells, which have physiological and pharmacological properties typical of mossy fiber transmission, namely high sensitivity to presynaptic metabotropic glutamate receptor agonists, steep frequency-dependent facilitation, and NMDA receptor-independent long-term potentiation (Gutierrez, 2000; Walker et al. 2001, 2002). Moreover, GABA_A receptors occur within mossy fiber synaptic clefts, and the subcellular distribution of GABA is similar to that of glutamate, implying localization to vesicles (Bergersen et al. 2003), even though the vesicular transporter VGAT has not been described in these synapses (Chaudhry et al. 1998).

Here we show that both Zn²⁺ and GABA are contained within the same mossy fiber synaptic varicosities. Prompted by evidence for co-localization of Zn²⁺ and GABA, we asked whether putative mossy fiber GABA_A receptor-mediated responses are modulated by endogenous Zn²⁺. We also asked whether, under similar conditions, more distant GABA_A receptors recruited by alternative stimuli are also able to detect endogenous Zn²⁺.

METHODS

Immunohistochemistry

We used a modification of Timm's histochemical method for Zn²⁺ staining (Seress and Gallyas, 2000) together with post-embedding

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immunogold labeling for GABA. Two adult male Sprague-Dawley rats were anesthetized with urethane (1.5 g/kg ip) and perfused over 1 min with a buffered Na_2S solution followed by 3% glutaraldehyde in 0.1% phosphate-buffered saline (PBS, pH 7.4, 20 min) and again with Na_2S solution (15 min). After dissection, brains were postfixed in 3% glutaraldehyde for 2 h on ice. Vibratome sections (50 μm) were washed in Tris buffer (pH) 7.4 and placed in tungstate physical developer (pH 5.5, 15 min) at room temperature. The reaction was stopped by placing the sections into 1% sodium thiosulphate (1 min). After final rinsing in Tris buffer, sections were osmicated (0.5% for 15 min), dehydrated, and embedded in Araldite according to standard protocols. For EM immunogold labeling, ultrathin sections (50 nm) were collected on pioloform-coated single-slot nickel grids. Grids were then mounted in a grid support plate and etched for 25 min in 1% periodic acid in distilled water. After washing in distilled water (3×5 min) grids were exposed to 2% sodium metaperiodate (25 min), rinsed in distilled water, and preincubated in incubation medium (IM) consisting of 1% bovine serum albumin and 10% fetal calf serum in PBS (30 min). Sections were then incubated with a rabbit anti-GABA antibody (1:4,000 in IM; A2052, Sigma) overnight at 4°C. After thorough washing (4×10 min in PB) and preincubation in IM (30 min), the secondary antibody (goat anti-rabbit IgG coupled to 10 nm gold particles; Sigma G-7402) was applied at a dilution of 1:100 in IM for 4 h at 37°C. Preparations were washed subsequently in PB (5×10 min) before final rinsing in double-distilled water. The sections were contrasted with uranyl acetate (4 min) and Reynold's lead citrate (50 s) according to standard EM methods. Preparations were examined using a Philips 201C electron microscope. Control sections from which the primary antibody was omitted showed no immunolabeling.

Electrophysiology

For the electrophysiology experiments, transverse hippocampal slices (350 μm) were obtained from 3 to 4 wk-old guinea pigs killed by cervical dislocation. Slices were stored in an interface chamber for ≥ 1 h prior to transfer to a submersion recording chamber. The storage and perfusion solution contained (in mM) 119 NaCl, 2.5 KCl, 1.3 MgSO_4 , 2.5 CaCl_2 , 26.2 NaHCO_3 , 1 NaH_2PO_4 , and 11 glucose, gassed with 95% O_2 -5% CO_2 (23–25°C). Two extracellular stimulating electrodes were used to evoke monosynaptic IPSCs. One was positioned in stratum radiatum of CA3 to activate local interneurons.

The other electrode was positioned in s. granulosum of the dentate gyrus. Its position was adjusted until an extracellular field potential recorded in CA3 showed an excitatory postsynaptic potential (EPSP) with a waveform typical of a mossy fiber response, which increased >2.5 -fold upon increasing the stimulation frequency from 0.05 to 1 Hz (Walker et al. 2001). The slice was discarded if this criterion was not met. Once a putative mossy fiber EPSP was identified, a whole cell recording was obtained from a CA3 pyramidal neuron under infra-red differential interference contrast imaging. The pipette solution contained (in mM) 135 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 2 MgATP, 0.3 Na_3GTP , and 5 QX314 Br (pH 7.2, osmolarity 295 mosM). AMPA/kainate and NMDA receptors were blocked with 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulphonamide disodium (NBQX, 20 μM) and D-2-amino-5-phosphonovalerate (APV, 50 μM) respectively. Currents were acquired with an Axopatch 1D amplifier (Axon Instruments), and records were filtered at 1 kHz and digitized at 2–5 kHz. The access resistance, monitored throughout the experiments using a voltage step, was <20 M Ω , and results were discarded if it changed by $>20\%$. Monosynaptic IPSCs evoked by dentate gyrus stimulation at 0.02 Hz were reversibly depressed $>40\%$ by 2-amino-4-phosphonobutyric acid (L-AP4, 10 μM), consistent with the selective sensitivity of mossy fiber synapses in guinea pigs (Yamamoto et al. 1983). At the end of the experiments, IPSCs were confirmed to be GABAergic by the addition of 100 μM picrotoxin.

N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), NBQX, APV, and picrotoxin were obtained from Tocris Cookson (Bristol, UK). A stock solution of calcium ethylenediamine tetra-acetate (Ca-EDTA) was prepared by adding $\text{Ca}(\text{OH})_2$ to ethylenediamine tetra-acetic acid in a 2:1 molar ratio. Statistical comparisons were made using Student's paired or unpaired *t*-test.

RESULTS

The mossy fiber varicosities in CA3 s. lucidum showed abundant co-localization of Zn^{2+} and GABA labeling (Fig. 1). GABA-immunogold labeling was mainly located at presynaptic vesicles, whereas the electron dense precipitate of the Zn^{2+} staining was most abundant close to pre- and postsynaptic sites of the synaptic cleft (Fig. 1) (see also Seress and Gallyas,

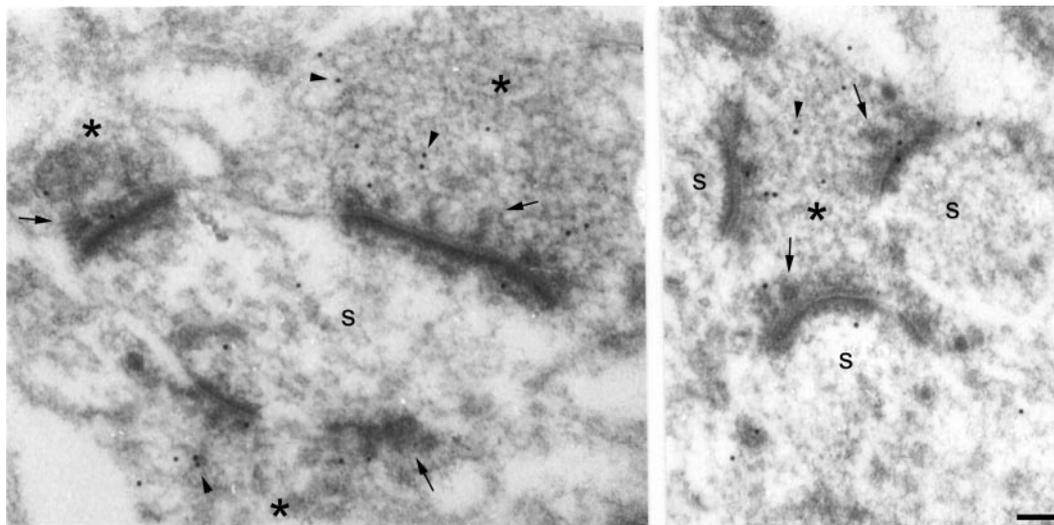


FIG. 1. Mossy fibers contain both GABA and Zn^{2+} . Electron micrographs from adult rat brain of mossy fiber varicosities (*) double stained for Zn^{2+} histochemistry and GABA immunolabeling. The electron dense reaction product of the Zn^{2+} staining (Timm's labeling) is apparent close to the synaptic clefts (\rightarrow), GABA-immunolabeling, which was visualized by a secondary, 10-nm gold-coupled antibody (arrowheads) is present at synaptic vesicles throughout the presynaptic terminals; s, postsynaptic spines. Scale bar: 100 nm.

2000). Labeling over areas where no GABA-immunoreactivity was expected (glial cells or glutamatergic varicosities in s. radiatum) did not exceed background levels observed over blood vessels or plain resin. The specificity of GABA antibody binding in rat hippocampus has been described further by Fabian-Fine et al. (2001). When mossy fiber profiles were analyzed systematically, the immunogold particle density was 9.5 ± 1.2 (SE) μm^{-2} ($n = 23$), as compared to a background density of 3.1 ± 0.9 μm^{-2} ($n = 23$ sampling areas matched for area; $P = 0.0001$, unpaired t -test). We thus conclude that mossy fibers labeled by the Timm's reaction for ionic Zn^{2+} are immunoreactive for GABA. A separate study by (Bergersen et al., 2003) has shown that the spatial distribution of GABA-immunoreactivity within hippocampal mossy fiber boutons is similar to that of glutamate but different from that of the nonreleased glutamine, consistent with an association with vesicles.

Although the EM data argue strongly for co-localization of GABA and Zn^{2+} within the same mossy fiber boutons, there is no evidence that endogenous Zn^{2+} modulates GABAergic transmission at this synapse. We therefore examined monosynaptic GABAergic IPSCs evoked in CA3 pyramidal neurons by stimulation in the dentate gyrus and compared them to IPSCs evoked by s. radiatum stimuli, designed to activate local interneurons. We used tissue from guinea pigs to exploit the high sensitivity of mossy fiber transmission to the group III metabotropic glutamate receptor agonist L-AP4 as an identifying criterion for these synapses.

Once a stable IPSC was recorded, we applied TPEN (1 μM), which has a much higher affinity for Zn^{2+} ($K_D = 0.26$ fM) than for Ca^{2+} or Mg^{2+} ($K_D = 40$ μM and 20 mM respectively) (Arslan et al. 1985). Addition of TPEN to the perfusion solution resulted in a reversible $26.5 \pm 6.3\%$ enhancement of the dentate gyrus-evoked GABAergic signal ($n = 9$, $P < 0.05$). However, it had no effect on the signal elicited by stimulation in s. radiatum in the same neurons (Fig. 2, A and B; comparison between pathways: $P < 0.05$).

Thus the GABA_A receptors mediating dentate gyrus-evoked

IPSCs, but not s. radiatum-evoked IPSCs, are inhibited by endogenous Zn^{2+} . Because TPEN crosses cell membranes, this result does not distinguish between chelation of intracellular and extracellular Zn^{2+} (Arslan et al. 1985). We therefore applied a second method to chelate extracellular Zn^{2+} selectively: Ca-EDTA does not cross cell membranes and has a much higher affinity for Zn^{2+} than other extracellular cations (Vogt et al. 2000). Perfusion of Ca-EDTA (2 mM) reversibly increased the dentate gyrus-evoked GABAergic signal by $31.9 \pm 6.9\%$ ($n = 8$, $P < 0.01$) but had no effect on IPSCs elicited by stimulation in s. radiatum (Fig. 2, A and B; comparison between pathways: $P < 0.01$). Thus, two distinct methods of chelating extracellular Zn^{2+} produced a similar robust and pathway-specific enhancement of dentate-evoked IPSCs, consistent with removal of inhibition of GABA_A receptors by Zn^{2+} .

In a separate series of experiments, we further confirmed the pathway specificity of this effect by determining whether Zn^{2+} chelation modulated GABA_A receptor mediated transmission at synapses recruited by stimulation in s. pyramidale. These responses demonstrated large "all-or-nothing" signals with a rapid rise time, characteristic of multiple perisomatic synapses. In common with IPSCs evoked by stimulating in stratum radiatum these responses were unaffected by Zn^{2+} chelation with TPEN ($96.3 \pm 0.8\%$, $n = 3$; Fig. 2B).

Two explanations for the pathway-specific effect of Zn^{2+} chelation are either that the GABA_A receptors recruited by dentate gyrus stimulation are detecting a greater Zn^{2+} concentration or that they are more sensitive to Zn^{2+} . We therefore compared the sensitivity of IPSCs evoked by dentate gyrus stimulation and by s. radiatum stimulation to exogenous Zn^{2+} . Bath application of 10 μM Zn^{2+} reversibly depressed both GABAergic responses to a similar extent and with a similar time course ($n = 4$ for each pathway, $P = 0.9$ for difference; Fig. 3). Bath application of 3 μM Zn^{2+} also reversibly depressed both GABAergic responses: to $83 \pm 8\%$ for dentate gyrus-evoked IPSCs and $73 \pm 5\%$ for s. radiatum-evoked IPSCs ($n = 3$ for each pathway, $P = 0.1$ for difference). These

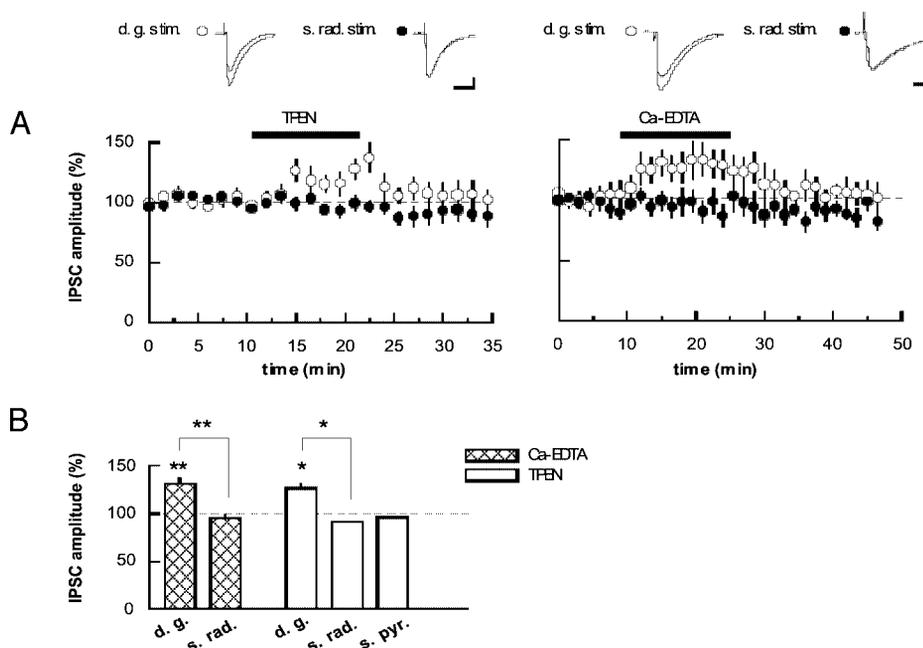


FIG. 2. Mossy fiber-like GABAergic inhibitory postsynaptic currents (IPSCs) in CA3 pyramidal cells are modulated by endogenous Zn^{2+} . A: Zn^{2+} chelation with *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN, $n = 9$, left) potentiates dentate-evoked mossy fiber-like IPSCs but not stratum radiatum-evoked IPSCs. Chelation with Ca-EDTA ($n = 8$, right) also enhances mossy fiber-like but not s. radiatum-evoked IPSCs. Insets: averages of 5 consecutive IPSCs before (thick traces) and after (thin traces) drug applications. Calibration bar: 100 pA, 50 ms. B: summary of the effect of Zn^{2+} chelation with Ca-EDTA or TPEN on GABAergic IPSCs evoked by stimulation in dentate gyrus, s. radiatum, or s. pyramidale. A significant effect was only seen on dentate-evoked IPSCs (** $P < 0.01$; * $P < 0.05$).

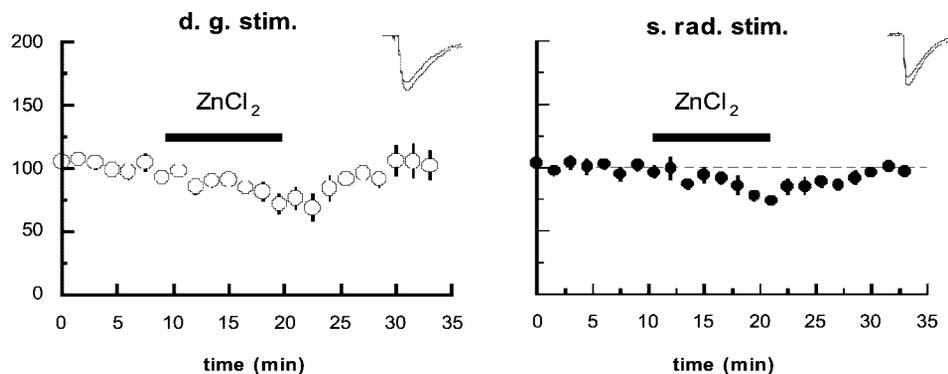


FIG. 3. IPSCs evoked by s. radiatum or s. granulosum stimulation are equally sensitive to exogenous Zn^{2+} . $ZnCl_2$ ($10 \mu M$) reversibly depresses IPSCs evoked by dentate gyrus (d.g., *left*) or stratum radiatum (s. rad., *right*) stimulation ($n = 4$, same neurons in both plots). *Insets*: averages of 5 consecutive IPSCs before (thick traces) and after (thin traces) $ZnCl_2$ application. Calibration bar: 100 pA, 50 ms.

results imply that the GABA_A receptors underlying both dentate gyrus- and s. radiatum-evoked IPSCs show similar sensitivity to Zn^{2+} .

DISCUSSION

The finding that GABA and Zn^{2+} can be co-localized in the same mossy fiber varicosities extends previous evidence for co-localization of glutamate and GABA immunoreactivity at mossy fiber varicosities (Sandler and Smith, 1991). Chelation of Zn^{2+} by either TPEN or Ca-EDTA enhanced GABAergic IPSCs evoked in CA3 pyramidal neurons by stimulating in s. granulosum of the dentate gyrus. These results lend support to the hypothesis that Zn^{2+} modulation of GABA_A receptors can occur in situ and therefore that pathological or developmental alterations in Zn^{2+} sensitivity are a potentially important aspect of plasticity of GABAergic transmission. Our results also demonstrate that this Zn^{2+} modulation is pathway specific.

TPEN and Ca-EDTA have complementary properties. TPEN shows high selectivity for heavy metals but is membrane-permeant, so it does not distinguish between chelation of extracellular and intracellular Zn^{2+} . Ca-EDTA, on the other hand, shows relatively less selectivity for Zn^{2+} but is membrane-impermeant. The finding that another pathway recorded simultaneously (s.-radiatum-evoked IPSCs) was unaffected argues against any significant disturbance of other extracellular cations. Both chelators produced a similar enhancement of dentate-evoked IPSCs, implying that they act on extracellular Zn^{2+} .

The enhancement of IPSCs by chelation demonstrated here was only detected with stimuli delivered to s. granulosum of the dentate gyrus, having previously verified that these stimuli evoked field EPSPs typical of mossy fibers. IPSCs evoked with this protocol have physiological and pharmacological properties typical of mossy fiber synapses (Walker et al. 2001). The finding that these IPSCs are modulated by endogenous Zn^{2+} , which occurs in higher extracellular concentrations in the vicinity of the mossy fiber pathway (Kay, 2003), lends further circumstantial support to the hypothesis that these IPSCs are indeed mediated by GABA release from mossy fibers (see also Gutierrez, 2000; Gutierrez and Heinemann, 2001; Gutierrez et al. 2003; Walker et al. 2002). In contrast, IPSCs evoked by stimulating either in s. radiatum or in s. pyramidale to excite local interneurons were unaffected. Although the stimuli used to recruit these GABAergic axons also activated glutamatergic fibers, any Zn^{2+} released under these conditions presumably reached too low a concentration to affect the GABA_A receptors.

The pathway specificity of our result may partly explain the failure to demonstrate an effect of mossy fiber stimulation on currents evoked by GABA uncaging in granule cells (Molnar and Nadler, 2001a). This design had been used to show a Zn^{2+} -mediated effect of very intense mossy fiber stimuli on exogenous NMDA receptor-mediated signals (Vogt et al. 2000). We also attempted, unsuccessfully, to affect the response to exogenous GABA, applied via pressure application, iontophoresis, or focal photo-uncaging with a UV laser, by delivering brief trains of stimuli to mossy fibers (A. Ruiz and D. DiGregorio, unpublished observations); this possibly reflects the poor spatial resolution of exogenous GABA application but could also be explained by the recent observation that very little Zn^{2+} may be released during synaptic activity (Kay, 2003). Indeed, all of the results of the present study can be explained by postulating that extracellular Zn^{2+} associated with mossy fibers exert a tonic inhibition of GABA_A receptors, which is relieved by chelation with TPEN or CaEDTA and that there is little dynamic modulation of this inhibition by synaptic release of Zn^{2+} with physiological patterns of stimuli.

The evidence that endogenous Zn^{2+} inhibits GABA_A receptors at mossy fibers is consistent with the immuno-EM data on the co-localization of Zn^{2+} and GABA. However, the electrophysiological data were obtained in guinea pig slices (to use L-AP4 sensitivity as a marker for mossy fiber synapses, which cannot be done in rat), while the anatomical data were obtained from rat tissue (because the fixation and staining protocols, and criteria used to identify mossy fiber boutons, have been developed in this species). Nevertheless, evidence for GABAergic transmission at rat mossy fibers has been reported, not only after seizures (Gutierrez, 2000; Gutierrez and Heinemann, 2001), but also in control tissue (Bergersen et al., 2003; Gutierrez et al., 2003), so it is highly likely that a similar modulation of GABA receptors occurs at mossy fibers in rats.

Does the Zn^{2+} sensitivity tell us anything about the GABA_A receptor subtypes activated by dentate gyrus stimulation? The sensitivity to low concentrations of Zn^{2+} implies that the receptors do not contain the γ subunit (Draguhn et al. 1990; Hosie et al. 2003) and/or that they contain the α_5 subunit (Burgard et al. 1996), which is abundant in the hippocampus (Sperk et al. 1997). However, our previous finding that mossy fiber-like GABA_A receptor-mediated IPSCs are sensitive to zolpidem (Walker et al. 2001) argues for receptors containing α_1 or α_2 subunits, together with γ subunits, a composition that renders the receptors relatively insensitive to Zn^{2+} . These two apparently conflicting findings can be resolved by hypothesizing that more than one receptor subtype is present at these

synapses. This is consistent with results obtained by titrating the Zn²⁺ sensitivity of GABA_A receptors in acutely dissociated pyramidal neurons (Tietz et al. 1999), although this has not been examined at individual synapses within CA3 pyramidal neurons.

The only previous evidence for an effect of Zn²⁺ chelation on GABAergic signaling was also reported in CA3 pyramidal neurons: Xie et al. (1994) described a decrease in the frequency of spontaneous large depolarizing potentials upon Zn²⁺ chelation in early postnatal slices due to a presynaptic synchronizing effect of Zn²⁺. Recent immunohistological data identify some other candidate synapses at which Zn²⁺-GABA interactions may occur. These include some synapses in the spinal cord and cerebellum where Zn²⁺, the Zn²⁺ transporter ZnT3, GABA, and/or glutamic acid decarboxylase are colocalized (Wang et al. 2001, 2002).

Modulation of GABA_A receptors by endogenous Zn²⁺ has profound implications for developmental and pathological processes, in particular, epilepsy. Experimental status epilepticus has been associated with a decreased Zn²⁺ sensitivity of GABA_A receptors (Banerjee et al. 1999; Kapur and Macdonald, 1997) while kindling and chronic human epilepsy increases Zn²⁺ sensitivity (Buhl et al. 1996; Gibbs et al. 2000; Shumate et al. 1998). Some of these changes may be explained by altered expression of subunits that affect the sensitivity of receptors to Zn²⁺ (Brooks-Kayal et al. 1998). The present results represent an important step toward determining whether a pro- or anti-epileptogenic role can be attributed to these changes. Ultimately, however, the pathological role of Zn²⁺ in modulation of GABA_A receptors will need to be seen in the context of its wider effects on NMDA receptors, glutamate transporters, and other signaling molecules.

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