Regionally Selective Blockade of GABAergic Inhibition by Zinc in the Thalamocortical System: Functional Significance

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1Department of Neurology, 2Department of Physiology, and 3Department of Anatomy, Medical College of Virginia of Virginia Commonwealth University, Richmond, Virginia 23298-0599; and Divisions of Neurology and Neuroscience, 4Department of Pediatrics, University of Pennsylvania School of Medicine and 5Pediatric Regional Epilepsy Program of the Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania 19104-4318

Gibbs, John W. III, Yun-Fu Zhang, Melissa D. Shumate, and Douglas A. Coulter. Regionally selective blockade of GABAergic inhibition by zinc in the thalamocortical system: functional significance. J. Neurophysiol. 83: 1510–1521, 2000. The thalamocortical (TC) system is a tightly coupled synaptic circuit in which GABAergic inhibition originating from the nucleus reticularis thalami (NRT) serves to synchronize oscillatory TC rhythmic behavior. Zinc is colocalized within nerve terminals throughout the TC system with dense staining for zinc observed in NRT, neocortex, and thalamus. Whole cell voltage-clamp recordings of GABA-evoked responses were conducted in neurons isolated from ventrobasal thalamus, NRT, and somatosensory cortex to investigate modulation of the GABA-mediated chloride conductance by zinc. Zinc blocked GABA responses in a regionally specific, noncompetitive manner within the TC system. The regional levels of GABA blockade efficacy by zinc were: thalamus > NRT > cortex. The relationship between clonazepam and zinc sensitivity of GABA_A-mediated responses was examined to investigate possible presence or absence of specific GABA_A receptor (GABAR) subunits. These properties of GABARs have been hypothesized previously to be dependent on presence or absence of the γ2 subunit and seem to display an inverse relationship. In cross-correlation plots, thalamic and NRT neurons did not show a statistically significant relationship between clonazepam and zinc sensitivity; however, a statistically significant correlation was observed in cortical neurons. Spontaneous epileptic TC oscillations can be induced in vitro by perfusion of TC slices with an extracellular medium containing no added Mg2+. Multiple varieties of oscillations are generated, including simple TC burst complexes (sTBCs), which resemble spike-wave discharge activity. A second variant was termed a complex TC burst complex (cTBC), which resembled generalized tonic clonic seizure activity. sTBCs were exacerbated by zinc, whereas cTBCs were blocked completely by zinc. This supported the concept that zinc release may modulate TC rhythms in vivo. Zinc interacts with a variety of tonic conductances, including GABAR currents, N-methyl-D-aspartate (NMDA) receptor currents, and transient potassium (A) currents. d–2-amino-5-phosphonovaleric acid and 4-aminopyridine blocked both s- and cTBCs in TC slices. Therefore NMDA and A current-blocking effects of zinc are insufficient to explain differential zinc sensitivity of these rhythms. This supports a significant role of zinc-induced GABAR modulation in differential TC rhythm effects. Zinc is localized in high levels within the TC system and appears to be released during TC activity. Furthermore application of exogenous zinc modulates TC rhythms and differentially blocks GABARs within the TC system. These data are consistent with the hypothesis that endogenously released zinc may have important neuromodulatory actions impacting generation of TC rhythms, mediated at least in part by effects on GABARs.

INTRODUCTION

Zinc is present throughout the brain in nerve terminals (Haug 1967; Wensink et al. 1988) contained within synaptic vesicles (Ibata and Otsuka 1969). This zinc can be released in concentrations as high as 100–300 μM during neuronal activity (Assaf and Chung 1984). Excessive levels of zinc have been proposed to be released during intense neuronal firing and could contribute to seizure discharge activity (Assaf and Chung 1984) with elevated levels associated with metabolic disorders and epileptic seizures. Although several studies have investigated both the normal and pathological roles of zinc release in the hippocampus (Brooks-Kayal et al. 1998; Buhl et al. 1996; Gibbs et al. 1997a; Xie and Smart 1993), no reports to date have detailed the effects of zinc in the TC system and the possible role of this cotransmitter in the modulation of TC rhythms. The TC system contains zinc in the cortex and thalamus, and particularly strong staining of zinc-containing terminals is evident in the nucleus reticularis thalami (NRT) (Haug 1973) (see also Fig. 1). The TC system is a tightly interconnected synaptic circuit consisting of the reciprocally connected cortex, thalamus, and an interposed GABAergic nucleus, NRT, which provides inhibitory innervation onto thalamus. GABAergic inhibition originating in NRT and impinging onto thalamus synchronizes and drives both normal and oscillatory TC activity, including sleep spindles, and the spike-wave discharges (SWDs) of Generalized Absence (GA) epilepsy (reviewed in Steriade and Llinás 1988).

Synchronously released zinc may serve as a neuromodulator, interacting with various ligand- and voltage-gated conductances to modify cellular responses. Zinc effects on GABARs, N-methyl-D-aspartate (NMDA) receptors, and voltage-gated K+ channels have all been described (Harrison and Gibbons 1994; Westbrook and Mayer 1987). However, the actual role of zinc in modulating function within the CNS is poorly understood. Zinc modulatory effects on GABA-mediated transmission have been demonstrated to be both pre- and postsynaptic in nature. Postsynaptically, zinc blockade of GABARs has been investigated in recombinant receptor systems and has been shown to depend on the subunit conformation of the receptor complex (Knoflach et al. 1996; Saxena and MacDonald 1994, 1996; Smart et al. 1991; White and Gurley 1995).
In addition, multiple putative zinc binding sites have been characterized within GABARs (Fisher and Macdonald 1998; Wang et al. 1995; Wooltorton et al. 1997). Given the distinct developmental and regional distribution of GABAR subunits in the brain (Laurie et al. 1992; Wisden et al. 1992), differential sensitivity of GABAR responses to zinc could have significant physiological consequences in the generation and synchronization of TC rhythms.

In the present study, whole cell patch recordings were conducted in acutely isolated neurons to investigate the modulatory effects of zinc on GABA-evoked responses in somatosensory cortex, ventrobasal thalamus, and NRT. Additionally, extracellular field potential recordings were performed to examine the effects of zinc on spontaneous TC rhythms. A preliminary report of these findings has been published in abstract form (Gibbs et al. 1996b).

**METHODS**

**Acute isolation of neurons**

Experiments were conducted on neurons acutely isolated from adult rat somatosensory cortex, ventrobasal thalamus, and NRT using methods that have been previously described (Gibbs et al. 1996a,b, 1998; Oh et al. 1995). The brain was dissected and placed in a 4°C chilled, oxygenated (95%O2-5%CO2) artificial cerebrospinal fluid (ACSF) solution composed of (in mM) 201 sucrose, 3 KCl, 1.25 NaHPO4, 2 MgCl2, 2 CaCl2, 26 NaHCO3, and 10 glucose. Coronal TC slices (400 μm) were cut and incubated for ≥1 h in an oxygenated medium containing (in mM) 120 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 25 glucose, and 20 piperazine-N,N9-bis[2-ethanesulfonic acid] (PIPES); with the pH adjusted to 7.0 with NaOH at 32°C. Slices were enzymed in 3 mg/ml Sigma protease XXIII in PIPES, thoroughly rinsed, and incubated another 30–60 min in PIPES medium before dissociation. The somatosensory cortex, thalamus, and NRT were visualized using a dark field microscope and microdissected out, especially making certain not to contaminate neuronal plates containing thalamic and NRT cells, which lie in very close proximity to one another. Chunks (1 mm3) then were cut from each area, and cells were dispersed mechanically by trituration of the chunks through Pasteur pipettes of decreasing bore sizes. The resulting cell suspension then was plated onto 35-mm culture dishes in N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid (HEPES) medium composed of (in mM) 155 NaCl, 3 KCl, 1 MgCl2, 3 CaCl2, 0.0005 tetrodotoxin, and 10 HEPES-Na+, with pH adjusted to 7.4 with NaOH.
Voltage-clamp recordings in isolated neurons

The intracellular (pipette) solution contained (in mM) 100 trizma phosphate (dibasic), 28 trizma base, 11 ethylene glycol bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 2 MgCl₂, and 0.5 CaCl₂, with pH adjusted to 7.35 with NaOH. Whole cell patch-clamp recording experiments were conducted on a Nikon inverted microscope equipped with Hoffman modulation contrast optics. Electrodes (4–8 MΩ) were pulled on a Narishige PP-83 microelectrode two-stage puller using thin-walled borosilicate capillary glass (WPI, Sarasota, FL). The pipette solution also contained an intracellular ATP reconstitution consisting of 10 mM Mg²⁺-ATP and 22 mM tris-phosphocreatinine 22. The intracellular ATP maintenance solution was used to fill the shank of the electrode but was omitted from the solution that was used to back fill the tip of the electrode. Recordings were amplified using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA) and filtered at 5 kHz with a 4-pole Bessel filter before digitization. All data were displayed on a chart recorder on-line (Gould, Model 2107, Cleveland, OH; frequency response DC-50 Hz) and stored on videotape after digitization (at 44 kHz) with a PCM interface (Neurodata Instrument, New York, NY). Data were played back on a chart recorder with a frequency response of DC-25 kHz (Astro-Med DASH IV, Warwick, RI).

Drug concentrations and method of application

GABA was prepared as a 10 mM stock solution in HEPES solution. Zinc first was dissolved in distilled water at 100 mM and then diluted to the final concentration in the HEPES medium, whereas clonazepam first was dissolved in dimethyl sulfoxide (DMSO) at 100 mM and then diluted to the final concentration in HEPES medium. The maximum concentration of DMSO used in cellular perfusions was <0.001% and has been shown not to alter GABA responses (Oh et al. 1995). The applied drug concentrations were as follows: GABA (Sigma, St. Louis, MO), 1 μM to 10 mM; clonazepam (Sigma), 100 mM; and zinc, 1–300 μM (Sigma). Solution changes were accomplished using a 13-barrel modified “sewer pipe” perfusion technique (Gibbs et al. 1997b) in which several solutions flowed out of parallel Teflon tubes (0.2 mm ID) in a laminar fashion. Rapid (40–200 ms) and complete solution changes at a constant flow rate then were effected by moving the tube assembly laterally in relation to the neuron under study. No cross-contamination was ever evident. After breaking the seal to institute whole cell recording mode and allowing ~2–5 min to pass to stabilize leak currents (0 to ~200 pA), GABA was applied for 4–6 s and washed out with control external solution for 30–40 s. The cell was pretreated with test drugs without GABA for 50–60 s and then test solutions were applied together with GABA. Drug effects with clonazepam and zinc were expressed as percentage effect on GABA-evoked outward currents, recorded at a V₁₀₀₀ of −24 mV. Experiments were performed at 22–24 °C.

Extracellular recording

TC slices were cut, and the presence of adequate TC connections verified using a dark field microscope as described previously (Agmon and Connors 1991; Coulter and Lee 1993; Zhang and Coulter 1996; Zhang et al. 1996a,b). Slices were transferred to an incubator containing ACSF [composed of (in mM) 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 0 MgCl₂, 2 CaCl₂, 26 NaHCO₃, and 10 glucose] where they were kept in warm (34°C) oxygenated medium. Before recording, slices were transferred to an interface recording chamber and perfused with warmed (34°C) oxygenated (95% O₂, 5% CO₂) ACSF. Insulated tungsten electrodes were positioned in TC slices with one electrode in the thalamus and one in the cortex to confirm generalization. Signals were recorded differentially with an AC-coupled four-channel amplifier at a gain of 1,000, with a ground electrode located nearby in the bathing medium. Signals were bypass filtered at 10–3,000 Hz. Data were displayed during an experiment on a four-channel chart recorder (AstroMed Dash IV, Warwick, RI) and digitized (at 22 kHz) and stored on videotape for later analysis using a PCM interface (Neurodata Instrument, New York, NY).

Drug application to TC slices

Zinc (100–300 μM), 4-aminopyridine (4-AP; 50 μM to 1 mM), and N,N,N',N'-tetraacetic acid (TETA; 10 μM) were dissolved into the ACSF and bath applied. A 15- to 30-min control period of stable TC activity was recorded, followed by a 10- to 30-min period of drug exposure and a 1-h wash in each experiment. Only reversible effects were analyzed.

Staining

Rat brain tissue was placed in a 1.2% Na₂S solution in a sodium phosphate buffer for 20–30 min and then transferred to a solution containing 1% paraformaldehyde and 1.25% glutaraldehyde in a sodium phosphate buffer and refrigerated overnight. The tissue then was placed in a 30% sucrose solution in a sodium phosphate buffer for 2–4 h, frozen in isopentane cooled with liquid nitrogen, and stored at −70°C until sectioning. Tissue was then cut on a cryostat into coronal 25-μm sections containing ventrobasal thalamus, somatosensory cortex, and NRT, melted onto gelatin-coated slides, and stored at −70°C. Sections then were thawed, hydrated, and developed in Timm’s stain (Haug 1973). Slide-mounted sections then were dehydrated, placed in xylene, and cover slipped for histological examination.

Statistical analysis on GABA response curves

All values are expressed as means ± 1 SE. Differences between means were tested using Student’s t-test. Concentration/response curves were fitted by a Marquardt-Levenberg nonlinear least-squares routine, using either ORIGIN (MicroCal Software, Northampton, MA) or ALLFIT (De Lean et al. 1987). The significance of differences in best fit parameter values between curves was assessed using constrained simultaneous curve fitting testing the equality of parameters, and ALLFIT, as described in De Lean et al. (1987). This method involves testing for equality of parameters by examining the statistical consequences (a via F test) of forcing them to be equal.

Statistical analysis on extracellular fields

After a preexposure to a medium containing no added Mg²⁺, TC slices exhibited two main types of spontaneous TC bursting activities, termed simple TC burst complexes (sTBCs) and complex TC burst complexes (cTBCs), respectively (Coulter and Lee 1993; Zhang and Coulter 1996; Zhang et al. 1996a,b). sTBCs had similarities to SWDs with morphology, frequency, duration, and pharmacological sensitivity to anticonvulsant drugs resembling bursting activity observed in GA epilepsy (5–10 Hz, 2–10 s). cTBCs had characteristic electro-physiological similarities to generalized tonic-clonic discharges (GTCs) with high-frequency tonic firing followed by a period of phasic bursting lasting in total usually 30–90 s in duration. These rhythms also had a pharmacological sensitivity to anticonvulsant drugs similar to GTCs (Zhang and Coulter 1996). Zinc was bath applied to TC slices exhibiting spontaneous epileptiform rhythms to examine the functional effects of zinc in an in vitro slice preparation capable of supporting sTBCs and cTBCs.

Modulatory effects on extracellular thalamocortical rhythms were quantified as has been described previously (Zhang and Coulter 1996; Zhang et al. 1996a). At least a 30-min control period of activity was recorded before drug application, and in the last 15 min of the control period, the chart record was examined to ensure that the spontaneous TC burst activity was stable. The last 5 events before the solution change then were employed to measure the average duration and
amplitude of cortical bursts. The duration was measured as the time from onset of the activity to the end of the last burst in the complex and was measured in the cortical recording trace because the signal-to-noise ratio was higher than in the thalamic trace. The duration measurement also reflected the thalamic burst complex duration because cortical and thalamic activity was coupled tightly. The amplitude of cortical bursting was measured peak to peak at the maximal level during the tonic burst activity in the cortical trace. Each of these measures was taken for five sequential TC burst complexes, and then the average of these five measurements was used for comparison. After change to a drug-containing solution, the level of drug effect was monitored until a maximum stable effect was achieved, and then a 15-min peak effect exposure period was monitored to ensure activity was stable. At the end of this 15-min period, immediately before the wash solution change, the last five TC burst complexes were quantified as described in the preceding text. After washout of drug, slices were monitored until a stable washout effect was achieved and then measurements taken as described in the preceding text. For construction of the time series bar graphs in this series of studies (Fig. 5, B and D), the average measures of events in each bin was continuously monitored, and the measurements averaged per bin.

RESULTS

Timm’s staining in TC slices

Presence of the endogenous metal cation, zinc, can be detected using Timm’s stain for heavy metals (Haug 1973). Coronal slices of rat brain were stained using the Timm’s method to investigate the presence of zinc-containing terminals in the cortex, ventrobasal thalamus, and the NRT (Fig. 1). Figure 1 shows a low (2×) and higher power (4×) photomicrographs of a coronal section stained for the presence of zinc-containing synaptic terminals. Note the prominent zinc stained terminals in the NRT (double arrows) and thalamus (single arrow). Prominent zinc-containing terminals were also present in the cortex (triangle) as seen in the lower power view (A).

Noncompetitive blockade of GABA-evoked responses

Acutely isolated cortical neurons were voltage-clamped at −24 mV, and GABA 1 μM-10 mM was applied alone and concurrently with zinc 30 μM (Fig. 2) in order examine the nature of the blockade of GABA-evoked responses exerted by zinc. Figure 2 plots the zinc 30 μM block of currents evoked by increasing concentrations of GABA in acutely isolated cortical neurons (n = 7). Application of increasing concentrations of GABA or GABA concurrently with zinc resulted in sigmoidally shaped concentration-response curves with GABA 1 mM eliciting a maximal GABA-evoked response (Gibbs et al. 1996a,b; Oh et al. 1995). The best fit EC50s for these curves were 28.5 ± 3.9 μM and 40.3 ± 14.7 μM, respectively. These EC50 means were not significantly different. However, EC50s derived from 3 point curves are not robust. Application of GABA 10 mM with zinc 30 μM was insufficient to overcome inhibitory effects of zinc, resulting in a 25% reduction of the GABA response (maximal effect 74.4 ± 7.3%). The lack of change in the EC50 of GABA-evoked responses in the presence of zinc and the inability of the saturating concentration GABA 10 mM to alleviate the blockade exerted by zinc were consistent with a noncompetitive blockade of GABA_A responses by zinc, as seen in previous studies in cultured cortical neurons (Celentano et al. 1991; Legendre and Westbrook 1991). However, EC50s derived from 3 point curves are not robust and more data points could not be added in individual cells due to limitations in the perfusion apparatus, so possible mixed blocking effects of zinc (cf. Gingrich and Burke 1998) cannot be discounted.

FIG. 2. Traces and concentration response curves illustrating the mechanism of zinc blockade of GABA-evoked currents in cortical neurons. A: traces illustrating zinc (30 μM)-evoked currents. Note that GABA 10 mM was not sufficient to overcome the blockade exerted by zinc 30 μM. B: concentration/response relationship for GABA applied alone and concurrently with zinc (30 μM). The best fit EC50s for these curves were 28.5 ± 3.9 μM and 40.3 ± 14.7 μM, respectively. These EC50 means were not significantly different. However, EC50s derived from 3 point curves are not robust. Application of GABA 10 mM with zinc 30 μM was insufficient to overcome inhibitory effects of zinc, resulting in a 25% reduction of the GABA response (maximal effect 74.4 ± 7.3%). This supports noncompetitive blockade by zinc of GABA-evoked responses.
Regional differences in zinc blockade of GABA-evoked responses in TC neurons

Cortical, thalamic, and NRT neurons were voltage-clamped at \(-24\) mV, and GABA 10 \(\mu M\) was applied alone and concurrently with varying concentrations of zinc to examine possible regionally-selective differences in zinc blockade of GABA-evoked currents. GABA 10 \(\mu M\) was on the rising phase of the concentration-response curve, allowing augmenting effects of GABA modulators to be demonstrated, but still was sufficient to provide robust GABA currents, facilitating study of GABA antagonists. This allowed direct comparison of the modulatory effects of zinc and CNZ in individual neurons. Application of zinc in concentrations of 1–300 \(\mu M\) resulted in a concentration-dependent, sigmoidally increasing blockade of GABA-evoked currents in all cellular types examined. The data could be best fitted assuming a sigmoidal concentration/response relationship using the equation:

\[
\% \text{ Inhibition} = M_1 \cdot C^H/(C^H + IC_{50}^H)
\]

where \(M_1\) is maximal zinc effect, \(C\) is zinc concentration, \(H\) is the Hill coefficient, and \(IC_{50}\) is the zinc concentration at which half-maximal effect was evident.

Zinc was found to block GABA-evoked responses regionally in a differential fashion. The efficacy of zinc blockade order was thalamus > NRT > cortex (curves significantly different; \(F = 34.37, P < 0.001\)). At 10 and 100 \(\mu M\), zinc blocked GABA-evoked responses by 39.3 ± 5.7% and 68 ± 4.4% in thalamic neurons (\(n = 21\)), by 21 ± 2% and 47.4 ± 2.3% in NRT neurons (\(n = 45\)), and by 14.6 ± 1.5% and 32.3 ± 3.2% in cortical neurons (\(n = 39\)), respectively (all values significantly different, \(P < 0.01\), t-test). Zinc 300 \(\mu M\) blocked of GABA-evoked responses by 84 ± 2.5%, 60 ± 1.8%, and 50 ± 1.4% in thalamic, NRT, and cortical neurons, respectively (all values significantly different, \(P < 0.001\), t-test) (Fig. 3). In a subset of cortical neurons (<5%), zinc paradoxically weakly augmented GABA-evoked responses. These cells were not included in the analysis.

**FIG. 3.** Traces and concentration inhibition curves illustrating zinc blockade of GABA-evoked currents in thalamic, NRT, and cortical neurons. **A:** traces illustrating the blockade of GABA (10 \(\mu M\))-evoked currents by zinc (1–300 \(\mu M\)). Note that increasing concentrations of zinc produced an increase in the levels of block of the GABA-evoked current in all neurons; however, the level of the GABA blockade showed regional differences. **B:** plots of the zinc concentration/GABA blockade curves in thalamic, NRT, and cortical neurons. The efficacy order for zinc blockade was thalamus > NRT > cortex. At 10, 100, and 300 \(\mu M\) zinc blocked GABA-evoked currents by 39.3 ± 5.7%, 68 ± 4.4%, and 84 ± 2.5% in thalamic neurons, by 21 ± 2%, 47.4 ± 2.3%, and 60 ± 1.8% in NRT neurons, and by 14.6 ± 1.5%, 32.3 ± 3.2%, and 50 ± 1.4% in cortical neurons, respectively.

Correlation between zinc inhibition and CNZ augmentation of GABA-evoked currents in TC neurons

**FIG. 4.** Correlation between zinc inhibition and CNZ augmentation of GABA-evoked currents in TC neurons. **A–C:** relationship between zinc inhibition/CNZ augmentation regionally in the different TC neuronal types. The zinc/CNZ relationship did not show a significant correlation in NRT and thalamic neurons (\(R = 0.22, P = 0.47; R = -0.44; P = 0.39\), respectively) but did exhibit a statistically significant correlation in cortical neurons (\(R = -0.88; P = 0.001\)).
FIG. 5. Zinc effects on spontaneous simple (sTBCs) and complex TC burst complexes (cTBCs). A: effects of zinc 300 μM on sTBCs. Control. Traces illustrating thalamic (top) and cortical (bottom) sTBCs recorded in a rat TC slice. Effects of zinc 300 μM on these sTBCs. Note the marked increase in duration and increase in amplitude of these events. Wash: recovery from the effects of zinc. Note that both the duration and amplitude effects of zinc 300 μM were reversible. B: histograms quantifying the zinc effects on sTBCs from A. Effects of zinc 300 μM (applied during the bar over the top) on the duration of sTBCs (top) and maximal amplitude of cortical bursts (bottom). Note that zinc 300 μM reversibly increased both the duration and burst amplitude of sTBCs. C: effects of zinc (300 μM) on cTBCs. Traces illustrating thalamic (top) and cortical (bottom) cTBCs recorded in a rat TC slice. Note the long duration (1–2 min) and complex multiphasic nature of the events. Recordings of the effects of zinc 300 μM on these cTBCs. Note that zinc blocked these events. Wash: recovery from zinc exposure. D: histograms quantifying the zinc effect from C. Top graph, effects of zinc on the duration of cTBCs. Bottom graph, effects of zinc on the amplitude of cTBCs. Note the reversible abolishment of cTBCs during zinc exposure.
FIG. 6. The effects of D-APV on spontaneous simple (sTBCs) and complex TC burst complexes (cTBCs). A: effects of D-APV (50 μM) on sTBCs. Control: traces illustrating thalamic (top) and cortical (bottom) sTBCs recorded in a rat TC slice. D-APV 50 μM: effects of D-APV 50 μM on sTBCs. Note the reversible decrease in duration and increase in amplitude of these events. Wash: recovery from the effects of D-APV. B: histograms quantifying D-APV effects on sTBCs illustrated in A. Effects of D-APV 50 μM (applied during the bar over the top) on the duration (top) and maximal amplitude of cortical bursts (bottom) of sTBCs. Note that D-APV (50 μM) reduced the duration and increased the burst amplitude of sTBCs, and these effects were reversible. C: effects of D-APV (50 μM) on cTBCs. Control: traces illustrating thalamic (top) and cortical (bottom) cTBCs recorded in a rat TC slice. D-APV 50 μM: recordings of the effects of D-APV 50 μM on cTBCs. Note D-APV reversibly blocked these events. Wash: recovery from D-APV exposure. D: histograms quantifying the D-APV effect from C. Top: effects of D-APV on the duration and amplitude of the cTBCs in C. Note the abolishment of cTBCs during D-APV exposure. Bottom: D-APV effects on the maximal burst amplitude during the tonic firing phase of the cTBCs. Note that D-APV suppressed the average burst amplitude.
FIG. 7. 4-AP effects on spontaneous simple and complex TC burst complexes (sTBCs and cTBCs). A: effects of 50 µM 4-AP on sTBCs (left) and cTBCs (right). Control: traces illustrating thalamic (top) and cortical (bottom) sTBCs (left) and cTBCs (right) recorded in a rat TC slice. 4-AP 50 µM: effects of 4-AP 50 µM on s- and cTBCs. Note the reversible increase in duration and increase in amplitude of these events. Wash: recovery from the effects of 4-AP. B: histograms quantifying the 4-AP effects on sTBCs (left) and cTBCs (right) from A. Effects of 4-AP 50 µM (applied during the bar over the top) on the duration (top) and maximal amplitude of cortical bursts (bottom) of sTBCs and cTBCs. Note that 4-AP 50 µM reversibly suppressed the duration and the burst amplitude of both these events.
Relationship between zinc/benzodiazepine modulation in TC neurons

Cloning expression studies of the GABAR subunit determinants mediating differences in pharmacology have demonstrated a γ2 subunit dependence to zinc sensitivity of GABARs. Receptors containing a γ2 subunit are insensitive to zinc, while receptors lacking a γ2 subunit are potently blocked by zinc (Draguhn et al. 1990; Saxena and Macdonald 1994; Smart and Constanti 1990). Benzodiazepine (BZ) sensitivity of GABARs is also conferred by the γ2 subunit (Pritchett et al. 1989). To explore whether possibly regionally selective differences in the contribution of γ2 subunits to GABARs was responsible for the above-described differences in zinc sensitivity, we examined the correlation between zinc inhibition and CNZ augmentation of GABA-evoked responses in individual cortical, thalamic, and NRT neurons. Neurons were voltage-clamped at −24 mV, and GABA 10 μM was applied alone and concurrently with zinc 100 μM or the BZ, clonazepam (CNZ) 100 nM, to examine zinc blockade-CNZ augmentation of GABA-evoked responses in the same neuron. Figure 4 shows the relationship in TC neurons between zinc inhibition/CNZ augmentation. The zinc/CNZ relationship showed no statistically significant correlation in NRT and thalamic neurons (R = 0.22, P = 0.47; R = −0.44, P = 0.39, respectively), but a significant correlation was evident in cortical neurons (R = −0.88; P = 0.001).

Zinc effects on sTBC and cTBC rhythms

The modulatory effects of zinc on TC rhythms were examined in 15 slices, supporting either sTBC or cTBC rhythms. Bath applied zinc (300 μM) was found to have differential modulatory effects on sTBCs and cTBCs, respectively. Figure 5 shows an example of the neuromodulatory effects of zinc 300 μM on in vitro sTBC and cTBC rhythms. Bath applied zinc 300 μM enhanced sTBC rhythms (n = 8) (Fig. 5A). The histogram in Fig. 5B shows the zinc-induced amplification of sTBC rhythms, including an enhancement of both the duration and amplitude of sTBC events. The frequency of sTBCs increased from 1.4–5.0 events/min to 2.75–9.5 events/min in the presence of zinc. In contrast, bath applied zinc 300 μM completely abolished cTBC rhythms (n = 7) (Fig. 5C). Figure 5D shows a complete reduction in both the duration and amplitude of the cTBC events.

APV and 4-AP effects on sTBC and cTBC rhythms

Zinc has been shown to have potent neuromodulatory effects on a variety of in vitro sTBCs and cTBC rhythms. Like α-APV, bath applied 4-AP 50 μM-1 mM was found to have strong inhibitory effects on both sTBC and cTBC rhythms. Figure 6 shows an example of the neuromodulatory effects of 4-AP on in vitro sTBC and cTBC rhythms. Bath applied 4-AP 50 μM-1 mM attenuated both cTBC and sTBC rhythms (n = 13) (Fig. 6). The histograms (Fig. 7B) show a 50–80% reduction in both the duration and amplitude of sTBC and cTBC events at 50 μM, and there was complete abolition of these events during perfusion of 1 mM 4-AP (data not shown). Since s- and cTBCs were virtually never seen in the same slices (Zhang et al. 1996a), the reduced amplitude and duration of cTBCs shown in Fig. 7 are probably reflecting a reduction in cTBCs, and not a transformation of cTBCs into sTBCs. There is an inverse relationship between the duration of an epileptiform event, and the frequency of occurrence of these events (Zhang et al. 1996a). In keeping with this relationship, the smaller, shorter s- and cTBC events in 4-AP occurred at higher frequencies (Fig. 7A). Therefore the total time spent in epileptiform activity in control and 4-AP may be similar.

DISCUSSION

Zinc blocked GABAR-mediated responses in a regionally specific manner within the TC system, with an efficacy order of: thalamus > NRT > cortex (Fig. 3). No correlation was found between BZ augmentation and zinc sensitivity in thalamic and NRT neurons; however, a statistically significant relationship was observed in cortical neurons (Fig. 4). Zinc had differential effects on spontaneous low Mg2+-induced epileptiform TC activity in TC slices. Zinc increased the amplitude and prolonged sTBC events which resembled spike-wave discharges, while zinc blocked cTBCs, which more closely resembled generalized tonic-clonic seizures (Fig. 5). Bath application of NMDA and K+ channel blockers could not replicate the differential effects of zinc on sTBCs and cTBCs, supporting the hypothesis that regionally selective GABAergic effects of zinc (among several possible sites) may play an important role in modulating TC rhythms (Fig. 6 and 7).

Zinc modulation of GABA_A-evoked responses

The GABAR is a heterooligomeric ligand-gated protein receptor composed of differing combinations of α, β, γ, δ, ε, ρ, π and θ subunits (reviewed in Bonnert et al. 1999; Davies et al. 1997; Hedblom and Kirkness 1997; Macdonald and Olsen 1994; Sieghart 1995). Differential subunit expression within the pentamer confers unique pharmacological properties to the GABAR (cf. Bonnert et al. 1999; Davies et al. 1997; Draguhn et al. 1990; Pritchett et al. 1989; Saxena and Macdonald 1994; White and Gurrley 1995) and could have important functional consequences in generation and modulation of rhythms in the brain. In the TC system, clonazepam differentially enhanced GABA-evoked responses in certain areas, with an efficacy order of NRT⇒ cortex⇒thalamus (Gibbs et al. 1996a). Clonazepam also blocked both sTBC and cTBC TC rhythms (Zhang et al. 1996b). Modulatory effects on GABA-evoked responses in neurons are determined primarily by the subunit configuration of the GABAR complex within individual populations of cells. In cloning expression studies, it has been found that the
γ2 subunit must be coexpressed with α and β subunits for the resulting receptor to be BZ sensitive. Expression of the γ subunit also impacts the zinc sensitivity of these receptors (Draguhn et al. 1990; Saxena and Macdonald 1994), along with transforming the mechanism of inhibitory block from noncompetitive to competitive (Gingrich and Burke 1998).

GABARs show both regional and developmental alterations in subunit conformation in the rat brain (Laurie et al. 1992; Olsen et al. 1990; Wisden et al. 1992). GABARs consisting of α and β subunits have been shown to be potently blocked by zinc. The addition of the γ subunit with corresponding α and β subunits resulted in zinc-insensitive GABARs (Draguhn et al. 1990) while replacement of the γ with a δ subunit enhanced zinc sensitivity (Saxena and Macdonald 1994). However, cultured hippocampal neurons expressing “native” BZ-sensitive GABARs are sensitive to zinc (Legendre and Westbrook 1990; Westbrook and Mayer 1987). Zinc sensitivity of γ2-containing GABARs has recently been shown to depend on α subunit expression. GABARs containing α1-, in comparison to α2- and α3- subunits coexpressed with β and γ subunits were less sensitive to zinc (White and Gurley 1995). In addition, GABARs comprised of the α6β3γ2 subunits were more sensitive to zinc modulation than α1β2γ1 GABARs (Saxena and Macdonald 1996). Expression of the δ subunit may also contribute to zinc and BZ sensitivity of GABARs. GABARs containing a δ subunit (replacing or coexpressed with a γ2 subunit) exhibited higher zinc sensitivity than γ2-containing receptors lacking a δ subunit (Saxena and Macdonald 1994).

In situ hybridization studies showed mRNA expression patterns in rat ventrobasal thalamus which were strongly positive for δ and weakly positive for γ2 subunits. The neocortex was positive for δ and strongly positive for γ2 subunits. NRT was positive for γ2 and negative for δ subunit expression (Wisden et al. 1992). Strong immunolabeling for the γ2 subunit has also been demonstrated in NRT (Gutiérrez et al. 1994). Given the Saxena and Macdonald (1994) findings coupled with in situ studies, the “δ/γ subunit hypothesis” of zinc sensitivity would predict the GABA blockade efficacy order to be thalamus > cortex > NRT based on mRNA profiles and regional clonazepam sensitivity studies in the TC system (Gibbs et al. 1996a; Oh et al. 1995). However, the present study showed a zinc inhibition relationship of GABA-evoked responses to be thalamus > NRT > cortex (Fig. 3). Given the strong CNZ effects in NRT neurons, supporting high expression of γ2 subunits within GABARs (Gibbs et al. 1996a) and the intermediate zinc sensitivity effects observed in the present study, these results would argue against the δ and γ subunits as being the primary subunits determining zinc sensitivity of native GABARs within the TC system.

Physiological and functional consequences of zinc TC rhythmicity

Measurement of zinc levels in the hippocampus suggest that the concentration in the extracellular space may reach as high as 300 μM during synaptic activity (Assaf and Chung 1984). Therefore the zinc concentrations (300 μM) employed in the present study are comparable to what may be experienced in vivo under periods of intense neuronal firing characteristic of epileptiform behavior. In vivo studies have shown that intracerebral injection of zinc causes seizures (Pei and Koyama 1986), neuronal cell death (Lees et al. 1990), and increased cortical neuronal firing rates (Wright 1984). Following zinc (50 μM) application, cultured hippocampal neurons fired high-frequency bursts of action potentials (Mayer and Vyklicky 1989). In hippocampal slices, bath applied zinc elicited giant depolarizing potentials (GDPs) mediated by GABARs in hippocampal pyramidal neurons (Xie and Smart 1991). Induction of these GDPs was blocked by agents that selectively chelate zinc (Ben-Ari and Cherubini 1991). Like hippocampal mossy fibers, the TC system exhibits high levels of staining of zinc-containing terminals (Haug 1973). In the present study, zinc (300 μM) was bath applied to TC slices during spontaneous low Mg2+-induced epileptiform activity to investigate possible in vivo effects of endogenously released zinc on TC rhythmicity and epileptiform behavior (Coulter and Lee 1993; Zhang and Coulter 1996; Zhang et al. 1996a,b). Differential effects of zinc (300 μM) were observed on sTBC and cTBC rhythms. Zinc increased the amplitude and prolonged sTBCs, but blocked cTBCs (Fig. 5). This suggests that, if zinc is released in the TC system during periods of high-frequency action potential firing, it could have differential modulatory effects on various types of TC rhythms.

Zinc has been shown to modulate a variety of ligand-gated and voltage-sensitive neuronal channels (reviewed in Smart et al. 1994) including GABARs (Celentano et al. 1991; Legendre and Westbrook 1991), NMDA receptors (Westbrook and Mayer 1987), and an inactivating K* current (Ih) (Harrison et al. 1993). Differential modulatory actions of zinc on multiple ionic channels could have elicited the opposite effects of zinc 300 μM on sTBC and cTBC rhythms. To more conclusively determine which of the cellular effects of zinc were important in modulation of TC rhythms, the selective NMDA antagonist, d-APV, and selective Ia blocker, 4-AP, were bath applied to TC slices generating sTBC and cTBC rhythms to compare effects of these agents to zinc effects. While zinc (300 μM) increased and inhibited sTBC and cTBC rhythms, respectively, bath application of APV (50 μM) or 4-AP (50 μM-1 mM) _blocked_ both sTBCs and cTBCs (Fig. 6 and 7). These NMDA and Ia current blockers did not mirror the effects of zinc on TC rhythms. Therefore zinc effects on sTBCs and cTBCs were not exclusively due to modulation of NMDA or Ia channels (Fig. 6 and 7).

While d-APV and 4-AP both blocked all variants of TC rhythms, the differential effects of zinc on TC rhythms were more difficult to interpret. Zinc, d-APV, and 4-AP suppressed cTBC rhythms in a similar fashion. In vitro cTBC rhythms induced by a media containing no added Mg2+ have been shown previously to be predominantly a cortical trigger, triggered by a paroxysmal depolarizing shift, with no thalamic component necessary for generation of cTBC rhythmicity (Coulter and Lee 1993). Zinc blockade of cTBC rhythms by could be due to zinc effects on NMDA receptors in cortical neurons, as has been previously reported in cultured hippocampal neurons (Westbrook and Mayer 1987) (Fig. 5).

Zinc effects on sTBC rhythms did not resemble those of d-APV or 4-AP. sTBCs require an intact TC circuit for expression (Coulter and Lee 1993). They appear to be driven by an underlying thalamic oscillation synchronized by GABAergic inhibitory IPSPs from NRT (Coulter and Zhang 1996). These IPSPs then trigger the activation of a low-threshold calcium current (Ih) in thalamus (reviewed in Steriade and Llinás 1988;
McCormik and Bal 1997). Thalamic relay nuclei rely on at least two transient currents, $I_A$ and $I_T$, which regulate the cellular excitability of the thalamic neuronal membrane potential at rest, with both the $I_A$ and $I_T$ conductances deactivating by hyperpolarization and activated by depolarization (Huguenard et al. 1991). Zinc enhancement of sTBC rhythms may be due to a combination of effects: blockade of $I_A$ in cortex and thalamus, and “disinhibition” of GABAergic synaptic circuitry in NRT, increasing NRT output, which would in turn increase the amplitude of GABA$_B$-mediated IPSPs from NRT onto thalamus, increasing deactivation of the thalamic T current, and augmenting excitability (Fig. 3). This hypothetical effect of zinc on sTBCs is analogous to bicuculline’s effects in enhancing spontaneous oscillations within the ferret lateral geniculate slices (von Krosigk et al. 1993). Firstly, blockade of $I_A$ by zinc would tend to disrupt the delicate balance between the $I_A$ and $I_T$ currents in thalamic relay neurons. Removal of the hyperpolarizing influence of $I_A$ would induce more robust and faster activation of $I_T$, promoting activation of low-threshold spikes (LTS) in thalamus. Additionally, the regionally selective zinc blockade of GABA-evoked responses could impact the genesis of sTBC rhythms (Fig. 3). A “disinhibition” of the GABA$_A$-mediated intra-NRT inhibitory synaptic connections (Ulrich and Huguenard 1996; von Krosigk et al. 1993) by zinc could also contribute to the amplification of sTBC rhythms. This would increase the inhibitory GABAergic output from NRT onto thalamic relay neurons, thereby promoting the activation of $I_T$ (cf. Destexhe 1998; Kim et al. 1997), which serves as a critical amplifier underlying sTBC and SWD TC rhythms (Coulter et al. 1989).

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