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Zn²⁺ chelation improves recovery by delaying spreading depression-like events

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Abstract

We previously reported Zn²⁺ chelation improved recovery of synaptic potentials after transient oxygen and glucose deprivation in brain slices. Such an effect could be due to reduced accumulation of Zn²⁺ in postsynaptic neurons, or could also be due to prevention of the onset of spreading depression-like events. A combination of optical and electrical recording was used here to demonstrate that Zn²⁺ chelation is effective because it delays spreading depression-like events. If the duration of oxygen/glucose deprivation was sufficient to generate a spreading depression-like event, irrecoverable Ca²⁺-dependent loss of synaptic potentials occurred, regardless of Zn²⁺ availability. These results identify a key mechanism underlying protective effects of Zn²⁺ chelation, and emphasize the importance of evaluating spreading depression-like events in studies of neuroprotection.

Keywords

Zinc; spreading depression; CA1; hippocampal slice; oxygen glucose deprivation; ischemia

INTRODUCTION

Transient removal of oxygen and glucose is widely used as an *in vitro* model of aspects of ischemic brain injury [1]. When applied to brain slice preparations, relatively short periods of oxygen and glucose deprivation are used (often 10 min) and neuronal viability can be continuously monitored from the amplitude of evoked excitatory postsynaptic potentials (EPSPs). An initial suppression of EPSPs occurs within minutes due to accumulation of adenosine and A1 receptor activation [2], however this suppression is readily reversible following restoration of metabolic substrates. Longer periods of substrate removal can lead to persistent loss of EPSPs which is indicative of neuronal injury [3].

We recently showed that chelation of Zn²⁺ significantly enhanced recovery of EPSPs after a fixed 10 min period of oxygen and glucose deprivation in the hippocampal CA1 region of murine brain slices [4]. Other studies have also implicated Zn²⁺ accumulation in injury following substrate removal in rat hippocampal slices [5]. The brain contains a large amount of Zn²⁺ [6] and while levels of this cation are normally tightly regulated within neurons, excessive Zn²⁺ accumulation is toxic to neurons [7,8]. The beneficial effects of Zn²⁺ chelation could be due to preventing deleterious actions of Zn²⁺ on neuronal mitochondria or other intracellular targets in CA1 neurons [4].

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An additional possibility is that Zn^{2+} chelation interferes with the onset of spreading depression (SD)-like events in the brain slice model. SD is a coordinated depolarization of neurons and glia that can occur in a variety of experimental and pathological conditions including ischemia [9], and a form of SD can be readily initiated in brain slices by oxygen and glucose deprivation [10] (termed here OGD-SD). If metabolic substrates are not immediately available to restore ionic gradients, OGD-SD causes rapid neuronal injury [9], and these responses may therefore be primary causes of neuronal injury in many slice ischemia studies [11]. We recently showed that accumulation of Zn^{2+} can contribute to the initiation of SD-like events in brain slices [10], and in the present study have examined whether delay of these events by Zn^{2+} chelation may be sufficient to explain beneficial effects of this intervention.

MATERIALS AND METHODS

All procedures were performed in accordance with the National Institutes of Health guidelines for the humane treatment of laboratory animals and the Institutional Animal Care and Use Committee at the University of New Mexico. Methods for preparation of brain slices coronal were described as previously [10] using C57Bl/6 mice at 4-6 weeks of age of either sex. Experiments were performed on submerged slices (300 μm), continuously superfused at 2 ml/min at 32°C (except where indicated otherwise). OGD-SD was monitored by intrinsic optical signals generated from transmission of >590nm light. Extracellular measurements of slow DC potential shifts or field EPSPs (fEPSPs) evoked by Schaffer collateral stimulation were made from stratum radiatum of hippocampal area CA1. Electrical stimuli (70 μs , 0.1Hz) were at a stimulus intensity that generated a response 70% of maximal. The superfusion system allowed for rapid (~0.5min) bath solution exchange after OGD-SD.

Superfusion buffer contained (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4 , 1 MgSO_4 , 26 NaHCO_3 , 2 CaCl_2 , and 10 glucose, equilibrated with 95% O_2 /5% CO_2 . Ice-cold cutting solution contained (in mM): 3 KCl, 1.25 NaH_2PO_4 , 6 MgSO_4 , 26 NaHCO_3 , 0.2 CaCl_2 , 10 glucose, 220 sucrose, and 0.43 ketamine, equilibrated with 95% O_2 /5% CO_2 . For oxygen and glucose deprivation, buffer was modified by equimolar replacement of glucose with sucrose and equilibrated with 95% N_2 /5% CO_2 . Nominally Ca^{2+} -free solutions were prepared by equimolar replacement of Ca^{2+} with Mg^{2+} . *N,N,N',N'*-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) was obtained from Invitrogen and all other reagents were obtained from Sigma. TPEN was prepared fresh daily as a 20mM stock in 0.25% DMSO. Data are reported as mean $\pm$ standard error of the mean. Significant differences between group data were evaluated using unpaired Student's t-tests.

RESULTS

1. Irreversible loss of synaptic potentials following spreading depression-like events

Figure 1A shows the propagation of oxygen/glucose deprivation-induced spreading depression (OGD-SD) as assessed by intrinsic optical imaging. A propagating band of increased light transmission is a consequence of tissue swelling that accompanies the coordinated depolarization [12]. In this example, OGD-SD propagated from left to right at 3.03 mm/min and evoked field potentials were abolished coincident with passage of the SD wave front past the recording site. Figure 1B shows mean data from five such experiments. The mean onset time to OGD-SD was 12.7 $\pm$ 0.2min and normal buffer was reintroduced into the chamber within 30 seconds of the passage of OGD-SD past the microelectrode recording site. OGD caused an initial loss of fEPSPs (to 7.8 $\pm$ 1.0% baseline), and a transient period of recovery then occurred ~4min prior to the onset of OGD-SD (peak response of 51.8 $\pm$ 7.3% baseline). This transient recovery of fEPSPs has been described during anoxia and is thought

to be due to the accumulation of extracellular K^+ [13]. Coincident with passage of OGD-SD, fEPSPs were abolished (Fig. 1C), and even though normal buffer was restored quickly following the event, there were no signs of fEPSP recovery within the time frame of these experiments.

In a separate set of experiments ($n=7$), slices exposed to brief episodes of OGD (7 min) that were not sufficient to produce SD were compared with exposures sufficient to generate OGD-SD. The short exposures produced a transient loss of fEPSPs ($6.5 \pm 0.8\%$ baseline) that recovered to near baseline levels ($90.1 \pm 2.3\%$) after 15 min restoration of normal buffer. When the same slices were then subjected to OGD-SD (mean onset time of 10.2 ± 0.3 min) an irrecoverable loss of fEPSPs (to $3.5 \pm 0.5\%$ baseline) was observed, consistent with results shown in Figure 1B. These results are consistent with the hypothesis that the deleterious consequences of SD are responsible for the persistent loss of field potentials following transient OGD exposures.

2. Delay of spreading depression-like events by Zn^{2+} chelation

We recently showed that chelation of Zn^{2+} with TPEN ($KD_{Zn}=10^{-15.6}M$; $KD_{Ca}=10^{-4.4}M$ [14]) can inhibit the initiation of some forms of spreading depression in hippocampal slices [10]. That prior study identified conditions (including activation of A1 receptors with exogenous application of an A1 receptor agonist) where Zn^{2+} chelation was sufficient to prevent the onset of spreading depression generated by the Na^+/K^+ /ATPase inhibitor, ouabain. It was also shown that OGD-SD could be delayed or prevented by Zn^{2+} chelation [10]. We confirmed a small but significant delay in OGD-SD onset using a combination of intrinsic optical recording and DC recordings of slow potential shifts [9] under the recording conditions of the current study (no A1 agonists) (12.0 ± 0.5 vs 14.5 ± 0.4 min, control and TPEN, respectively, $n=6$, $p<0.001$). This difference appeared potentially large enough to explain beneficial effects of Zn^{2+} chelation, depending on the duration of the challenge with oxygen and glucose removal.

3. Lack of injury if Zn^{2+} chelation prevents OGD-SD during transient challenges

From the results above, a set duration (13min) of oxygen and glucose removal was chosen as a test challenge, to test whether beneficial effects of TPEN were due to preventing OGD-SD onset. With 13 min challenges, OGD-SD should always be generated in vehicle controls, while TPEN-exposed preparations should never experience OGD-SD during these exposures. Indeed, as shown in Figure 2, a 13min oxygen and glucose removal challenge in the presence of TPEN ($50\mu M$, 20min pre-exposure, $n=5$, open triangles) produced only a transient loss of fEPSPs (to $8.9 \pm 1.2\%$), followed by full recovery of fEPSPs to pre-stimulus levels (to $109.9 \pm 13.5\%$ baseline). This significant protection was due to TPEN, since in matched vehicle controls, OGD-SD was generated by this 13 min exposure and resulted in irrecoverable loss of synaptic potentials (filled circles).

A separate set of studies was conducted to monitor fEPSP responses to OGD in the presence of TPEN. Figure 2C shows that if SD is allowed to occur, there is no significant benefit from Zn^{2+} chelation. In these experiments, OGD was maintained until SD occurred in the presence of TPEN (onset 14.0 ± 0.4 min, $n=5$) and rapid restoration of normal buffer did not restore synaptic function ($6.0 \pm 0.5\%$ at 60 min). Together, these results imply that preventing Zn^{2+} accumulation is not sufficient to prevent neuronal injury following OGD-SD, but Zn^{2+} chelation can completely prevent neuronal injury by preventing the onset of SD during transient OGD exposures.

4. Contribution of Ca^{2+} to persistent neuronal injury after OGD-SD

A final set of experiments confirmed that excessive Ca^{2+} (rather than Zn^{2+}) accumulation following OGD-SD is the most likely contributor to neuronal injury following OGD-SD in slice (Fig 3). Effects of Ca^{2+} removal were tested by exposure to modified buffer lacking added Ca^{2+} and without addition of any chelators (nominally Ca^{2+} -free, estimated free Ca^{2+} ~ 4 μM [15]). Under the recording conditions used in studies above (300 μm slices at 32°C), SD could not be generated by OGD in nominally Ca^{2+} -free buffer ($n=3$), and alternative recording conditions were identified (350 μm slices at 35°C) that allowed reliable generation of OGD-SD in nominally Ca^{2+} -free buffer. Ca^{2+} was removed 5min prior to onset of OGD exposures, and Ca^{2+} -free conditions maintained for a further 5 min after OGD exposure. Under these conditions, SD was generated at $5.5 \pm 0.4 \text{ min}$ ($n=5$), compared with $6.4 \pm 0.6 \text{ min}$ ($n=5$) in normal buffer. After OGD-SD no recovery of fEPSPs was observed in slices with Ca^{2+} present throughout, but a substantial recovery of fEPSP amplitude (to $69.2 \pm 8.2\%$) was seen in slices where Ca^{2+} was not available during the passage of SD. These results contrast with the lack of effect of Zn^{2+} removal in slices where SD was allowed to occur (Fig 2C), and confirm that Ca^{2+} (rather than Zn^{2+}) is a main contributor to persistent loss of synaptic activity following SD events triggered by OGD.

DISCUSSION

Zn^{2+} accumulation is strongly implicated in ischemic neuronal injury, and there is significant interest in understanding the cellular and molecular mechanisms underlying Zn^{2+} toxicity [8,16,17]. Studies of dispersed neuronal cultures have identified pathways that contribute to Zn^{2+} toxicity during excessive glutamate receptor activation or challenge with oxygen and glucose deprivation (OGD) [18–21]. When OGD is undertaken in brain slices, deleterious Zn^{2+} dependent mechanisms that have been identified in dispersed cultures may be activated in neurons. However in brain slices, OGD also triggers a profound depolarization event that propagates slowly through the well-organized structure of the slice [9–11]. These events share many similarities with the phenomenon of spreading depression (SD), and can not be generated in dispersed cultures, as the very large extracellular volume prevents glutamate and K^+ levels accumulating to threshold concentrations for maintaining a feed-forward depolarization. The results of the present study indicate that beneficial effects of Zn^{2+} chelation in the brain slice OGD model can be attributed to prevention of the onset of OGD-SD. Other deleterious effects of intracellular Zn^{2+} accumulation may be activated in CA1 neurons during the OGD exposure, however preventing these effects is not sufficient to prevent the injury caused by massive Ca^{2+} accumulation if SD occurs during this challenge.

It is not fully understood how Zn^{2+} contributes to the onset of OGD-SD, but mitochondrial disruption is one possibility [4,10,22]. As a note, relatively high concentrations (0.4%) of the vehicle used here (DMSO) have been reported to delay the onset of SD induced by hypoxia [23]. However no delay was observed with the lower DMSO concentrations used here (0.25%, data not shown) and since this vehicle was included all experiments, DMSO effects are not expected to explain the delay of SD and subsequent recovery seen by TPEN.

In previous work, removal of Ca^{2+} following SD induced by OGD restored membrane potential [24]. Consistent with those results, removal of Ca^{2+} during OGD-SD allowed for partial recovery of fEPSPs, indicating that the Ca^{2+} overload during OGD-SD [10] is primarily responsible for the persistent depression of synaptic activity. The residual injury could be due to incomplete removal of Ca^{2+} , but since our previous study showed an enhanced recovery after OGD with combined removal of Zn^{2+} and Ca^{2+} [4], we attempted to determine whether the combined removal would enhance synaptic recovery following SD. However under these slice and recording conditions, we were not able to induce OGD-

SD with both Ca^{2+} and Zn^{2+} removal, as confirmed by a lack of a DC shift and intrinsic optical signals ($n=6$, data not shown). This is in agreement with our prior report under different recording conditions and conclusion that both cations contribute to SD initiation [10,22]. However, because of this block of OGD-SD it is currently unknown whether Zn^{2+} accumulation following SD can contribute with Ca^{2+} to neuronal injury. It is possible that in preparations that do not undergo SD, postsynaptic Zn^{2+} accumulation contributes to different forms of injury.

CONCLUSION

These results suggest that delay of the onset of OGD-SD can be sufficient to explain the beneficial effects of Zn^{2+} chelation in a slice model of ischemia. Such effects could be important for limiting the spread of ischemic injury *in vivo*. Additional deleterious effects of Zn^{2+} accumulation may also contribute significantly to injury, in other ischemia models or conditions where massive ionic disruptions associated with SD events are not the predominant causes of injury.

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Figure 1.

Onset of persistent synaptic inhibition coincident with OGD-SD. **A.** Monitoring propagation of SD by intrinsic optical imaging. Representative bright-field image (showing placement of electrodes) and montage illustrating OGD-SD propagation across CA1 hippocampal subfield. Scale bar 200 μm , image interval 1.5s, advancing wave front indicated by bright band. **B.** Loss of fEPSPs during OGD. OGD was continued in each slice until SD was visualized, and then the superfusate was rapidly switched back to normal buffer. A transient recovery of fEPSPs occurred immediately prior to OGD-SD, and was followed by persistent (>1hr) inhibition (n=5). *Inset:* Representative fEPSP traces at time courses depicted on mean data plot. **C:** Correlation between OGD-SD onset and loss of fEPSPs ($r^2 = 0.99$).

Figure 2.

Delay of OGD-SD by TPEN was responsible for recovery of fEPSPs. **A.** In these experiments, the duration of OGD was fixed (13 min), so that under vehicle conditions OGD always produced SD ($n=5$, circles) but the OGD duration was never long enough to generate SD in TPEN slices ($50\mu\text{M}$, $n=5$, triangles). fEPSPs were abolished in control, but fully recovered in TPEN. Lower panels show representative traces of fEPSPs during time points indicated in the plot. **B.** In contrast, if OGD was maintained until SD was generated in TPEN slices (average 14.03 ± 0.38 min, $n=5$) no recovery of fEPSPs was observed. *Inset:* Representative fEPSPs traces at times indicated on mean plot.

Figure 3.

Ca²⁺ influx was responsible for irrecoverable synaptic loss following OGD-SD. **A.** Slices superfused with Ca²⁺-free buffer during OGD (and 5min pre-, post-exposure) showed partial recovery of fEPSPs even after the generation of OGD-SD (squares), when compared with paired control slices which showed irrecoverable fEPSP loss (n=5, both groups). **B.** Representative fEPSP traces taken from time points indicated on mean plot.