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# Mild Acidosis Enhances AMPA Receptor-Mediated Intracellular Zinc Mobilization in Cortical Neurons

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Overactivation of glutamate receptors and subsequent deregulation of the intraneuronal calcium ( $Ca^{2+}$ ) levels are critical components of the injurious pathways initiated by cerebral ischemia. Another hallmark of stroke is parenchymal acidosis, and we have previously shown that mild acidosis can act as a switch to decrease NMDAR-dependent neuronal loss while potentiating the neuronal loss mediated by AMPARs. Potentiation of AMPAR-mediated neuronal death in an acidotic environment was originally associated only with  $Ca^{2+}$  dyshomeostasis, as assessed by  $Ca^{2+}$  imaging; however, intracellular dyshomeostasis of another divalent cation,  $Zn^{2+}$ , has recently emerged as another important co-factor in ischemic neuronal injury. Rises in  $Zn^{2+}$ , greatly contribute to the fluorescent changes of  $Ca^{2+}$ -sensitive fluorescent probes, which also have great affinity for  $Zn^{2+}$ . We therefore revisited our original findings (McDonald et al., 1998) and investigated if AMPAR-mediated fura-2 signals we observed could also be partially due to  $Zn^{2+}$  increases. Fura-2 loaded neuronal cultures were exposed to the AMPAR agonist, kainate, in a physiological buffer at pH 7.4 and then washed either at pH 7.4 or pH 6.2. A delayed recovery of fura-2 signals was observed at both pHs. Interestingly this impaired recovery phase was found to be sensitive to chelation of intracellular  $Zn^{2+}$ . Experiments with the  $Zn^{2+}$  sensitive (and  $Ca^{2+}$ -insensitive) fluorescent probe FluoZin-3 confirmed the idea that AMPAR activation increases  $Zn^{2+}$ , a phenomenon that is potentiated by mild acidosis. Additionally, our results show that selective  $Ca^{2+}$  imaging mandates the use of intracellular heavy metal chelators to avoid confounding effects of endogenous metals such as  $Zn^{2+}$ .

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## INTRODUCTION

Excitotoxicity is a key phenomenon that promotes neuronal demise in cerebral ischemia (1). Evidence cumulated in the past 30 years has linked ischemic neuronal death to the overactivation of glutamatergic ionotropic receptors (NMDA, N-Methyl-D-aspartate and AMPA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid), a process that leads to massive  $Ca^{2+}$  entry into neurons and activation of a plethora of  $Ca^{2+}$ -dependent injurious pathways. NMDAR associated channels are highly  $Ca^{2+}$  permeable and seem to play a prominent role in ischemic neuronal death. Most of

the AMPAR associated channels are  $Ca^{2+}$  impermeable; nevertheless, AMPAR activation is also able to mediate neurotoxic rises of intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) via indirect opening of the voltage sensitive  $Ca^{2+}$  channels (VSCC). Parenchymal acidosis is another key component of the injurious pathways set in motion by cerebral ischemia (2), as well as an important modulator of excitotoxicity. NMDAR-mediated currents are attenuated by moderate extracellular acidity in the range of pH 6.2-7.2 (3-4), and in vitro, reduction of extracellular pH below 6.5 reduces both glutamate and oxygen-glucose deprivation induced neurotoxicity

(5-7). While acidosis can reduce NMDAR-dependent neuronal loss, we previously have shown that lowering the extracellular pH can enhance AMPAR-mediated neuronal injury (8). Such enhanced toxicity originally was explained partially by increased  $[Ca^{2+}]_i$  deregulation. In the McDonald study, we also found that neurons challenged with AMPA (under non-desensitizing conditions) undergo a phase of delayed recovery of  $[Ca^{2+}]_i$  levels when washed in an acidic (pH 6.6) buffer. Using  $Ca^{2+}$  imaging experiments, this delayed recovery was attributed to deregulation of  $[Ca^{2+}]_i$  homeostatic mechanisms, as evaluation of  $Ca^{2+}$  influx by  $^{45}Ca^{2+}$  uptake showed that influx of the cation via VSCC was decreased by extracellular acidity.

Excitotoxic neuronal loss also has been linked to the dyshomeostasis of another divalent cation,  $Zn^{2+}$ . A large amount of data supports the idea that  $Zn^{2+}$  can in

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fact act not only as a cellular messenger, but also as a potent trigger of neuronal death through several mechanisms – including energy depletion, reactive oxygen species (ROS) generation, and mitochondrial dysfunction, reviewed in (9). Injurious  $[Zn^{2+}]_i$  rises have been linked to excitotoxic conditions such as transient global ischemia, head trauma, and epilepsy (10). Elevations of  $[Zn^{2+}]_i$  are due to a combination of influx of the cation via glutamate receptors and VSCC activation, as well as its release from intracellular sites. Intracellularly  $Zn^{2+}$  is loosely bound to proteins such as metallothioneins (MTs) and several studies have indicated that the cation can be mobilized from MTs under conditions of oxidative stress. Further studies have shown that  $Zn^{2+}$  also can be mobilized in a  $Ca^{2+}$ -dependent fashion from mitochondria (11–12). Interestingly, changes in the intracellular proton concentration appear to act as a co-factor in ROS-mediated  $[Zn^{2+}]_i$  rises as a decrease in pH rapidly destabilizes  $Zn^{2+}$  binding to MTs (13) and promotes an overall release of the cation. This phenomenon has now been confirmed in neurons, where mild acidosis has been found to dramatically increase  $[Zn^{2+}]_i$  rises induced by MT oxidation (12).

A very intriguing debate has recently emerged in the excitotoxic field: the “Calcium- Zinc dilemma” (9,12,14–16). While for many years excitotoxicity has been interpreted as a purely  $Ca^{2+}$ -dependent process, most of the evidence for the  $Ca^{2+}$ -dependency derives from experimental paradigms that made use of either  $Ca^{2+}$  chelators or intracellular  $Ca^{2+}$  imaging with  $Ca^{2+}$  sensitive fluorescent indicators. One problem with this “ $Ca^{2+}$ -centered” view comes from the fact that both  $Ca^{2+}$  fluorescent probes and chelators also bind  $Zn^{2+}$ , and in fact do so with higher affinity. The potential confounding effect of  $Zn^{2+}$  on  $Ca^{2+}$  imaging has been neglected, moving from the assumption that  $[Zn^{2+}]_i$  levels are very low. However, as mentioned above, a wealth of evidence has been gathered in recent years demonstrating that upon patho-

physiological conditions (i.e. oxidative and nitrosative stress; NMDAR activation and oxygen-glucose deprivation)  $[Zn^{2+}]_i$  rises can reach levels high enough to greatly interfere with fluorescent measurements of  $[Ca^{2+}]_i$  and/or chelation by divalent chelators (12,14,16–17). Thus, in this study, we critically re-examined our previous results (8) and investigated whether the AMPAR-mediated changes in fura-2 fluorescence signals that we originally interpreted as purely  $Ca^{2+}$ -dependent could be also linked to  $[Zn^{2+}]_i$  rises.

## MATERIALS AND METHODS

### Materials

FluoZin-3 AM, fura-2 AM, and hydroethidine (Het) were purchased from Molecular Probes (Invitrogen, Milan, Italy). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), and *N,N,N',N'*-Tetrakis-(2-pyridylmethyl)ethylene-diamine (TPEN) were obtained from Sigma-Aldrich (Milan, Italy). Tissue culture media and serum were from Gibco (Invitrogen, Milan, Italy). All other chemicals and reagents were obtained from common commercial sources.

### Animal Cell Cultures

All animal procedures were approved by the institutional animal care and use committee. Murine cortical cultures were prepared from embryonic CD1 mice and neurons plated upon astrocytic monolayers on poly-lysine + laminin coated cover slips as previously described (18).

### Fura-2, FluoZin-3, and HET Microfluorimetric Studies

Fluorescence imaging was carried out using an inverted microscope with a xenon lamp, filter wheel, at 40X, epifluorescence oil immersion objective and fluorescent cubes. For ratiometric fura-2 imaging, cultures were loaded in the dark, with 3  $\mu$ M of the acetoxymethyl ester form of the probe in a HEPES-buffered medium (HCSS) whose composition was (in mM): 120 NaCl, 5.4 KCl, 0.8  $MgCl_2$ , 20 HEPES, 15 glucose, 1.8  $CaCl_2$ , 10

NaOH, pH 7.4 for 30 min at 25°C, then washed in HCSS and kept in the dark for an additional 30 min, excitation was at 340 and 380 nm, with emission at 510 nm. For  $Zn^{2+}$  imaging, cells were loaded with the same protocol using 5  $\mu$ M of FluoZin-3 AM, excitation was at 490 nm and emission at 530 nm. Experiments were carried out at room temperature (25°C). Drugs were applied by bath application and removed through a rapid flow exchange system. Images were acquired with a 12-bit digital CCD camera and analyzed (after background subtraction from a cell-free region of the dish) with Metafluor 6.0 software (Universal Imaging, West Chester, PA, USA).

Oxygen radical production was monitored using the oxidation sensitive dye, HET. Stock HET (1 mg/mL) was prepared as previously described (11) in dry DMSO and stored in frozen aliquots for use within eight weeks. Cultures were loaded in the dark with 5  $\mu$ M HET in HCSS (45 min, 25°C). After loading, cultures were washed (4 $\times$ ) in a static bath of HCSS containing 5  $\mu$ M HET. Cells were excited at 510–560 nm and emission monitored at > 590 nm.

Fura-2, FluoZin-3, and HET fluorescence measurements for each cell (Fx) were normalized to either the fluorescence intensity (FluoZin-3 and HET) or 340/380 ratio (fura-2) for that cell at the beginning of the experiment (F0) and changes over time expressed as percentage increase over baseline.

### Experiment Replication and Statistics

All experiments reported represent at least three independent replications. Comparisons were obtained by Student-Newman-Keuls' test ( $P < 0.01$ ).

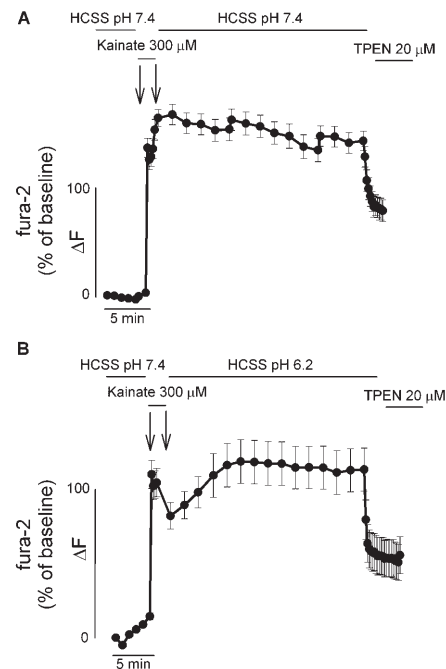
## RESULTS

We employed an experimental paradigm similar to the one used in our original study (8). Cortical neurons were loaded with the  $Ca^{2+}$ - (and  $Zn^{2+}$ -) sensitive ratiometric fluorescent probe fura-2 and fluorescence changes evaluated both during a 1 min exposure to 300  $\mu$ M kainate and in the washout phase

(30 min at pH 7.4). At the end of the 30 min washout phase, neurons were exposed for 5 min to the cell-permeant heavy metal (and high affinity  $Zn^{2+}$ ) chelator TPEN [20  $\mu$ M;  $K_d$   $10^{-26}$  (19)]. In a second set of experiments, fura-2 fluorescence changes were investigated in neurons that, after the kainate challenge at pH 7.4, were washed in an acidic buffer (pH 6.2) for 30 min and then exposed to TPEN. Surprisingly, both in the case of post-kainate washout at pH 7.4 and pH 6.2, we observed a delayed recovery in the fura-2 signal. This delayed recovery in fura-2 fluorescence was found to be largely reduced upon TPEN exposure (Figure 1A and B). When we evaluated the extent of the decrease in the fura-2 signal produced by the TPEN exposure, we found no statistical difference between the two conditions. TPEN can decrease fura-2 fluorescence by 58% (SEM  $\pm$  4.33) in neurons washed at pH 7.4, while the same maneuver at pH 6.2 produced a 53% (SEM  $\pm$  2.68) fluorescence drop. When analyzing these  $[Zn^{2+}]_i$  rises, one has to consider that potential differences could be difficult to be evaluated considering the high affinity for  $Zn^{2+}$  of fura-2 ( $K_d$  0.5–3 nM).

To better analyze AMPAR-mediated  $[Zn^{2+}]_i$  dyshomeostasis, we therefore loaded our cortical neurons with the  $Zn^{2+}$ -sensitive (and  $Ca^{2+}$ -insensitive) fluorescent probe FluoZin-3 (20). As with the fura-2 experiments, FluoZin-3 loaded neurons were imaged upon a brief excitotoxic pulse (300  $\mu$ M kainate for 1 min at pH 7.4) and during a 20 min washout period either at pH 7.4 or pH 6.2 (Figure 2A and B). Integral analysis of  $[Zn^{2+}]_i$  overload occurring in the washout period revealed that post-kainate  $Zn^{2+}$  dyshomeostasis is enhanced by extracellular acidosis (Figure 2C).

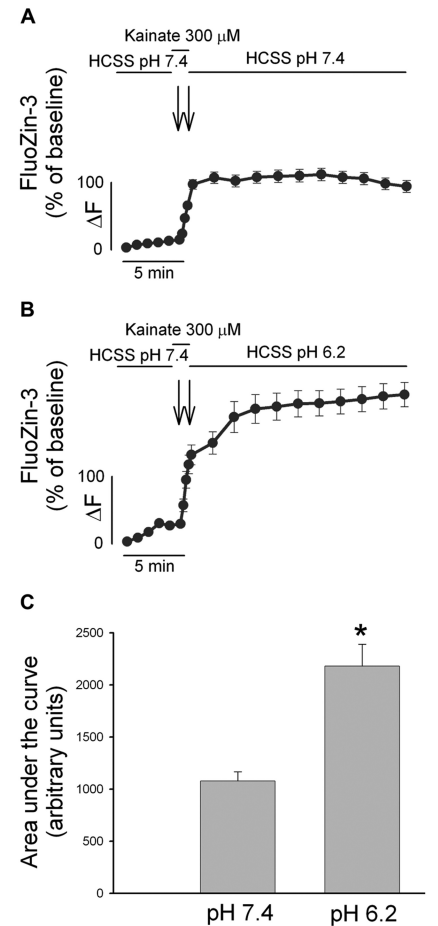
To elucidate the source of the  $[Zn^{2+}]_i$  rises triggered by kainate exposure, we performed a control experiment in which FluoZin-3 loaded neurons were challenged with kainate and washed in an acidotic buffer in the presence of the extracellular  $Zn^{2+}$  chelator  $Ca^{2+}$ -EDTA (20  $\mu$ M). Indicating that AMPAR activa-



**Figure 1.** AMPAR activation increases both  $(Ca^{2+})_i$  and  $(Zn^{2+})_i$  levels. A, B. Time course of  $Ca^{2+}$  and  $Zn^{2+}$ -dependent changes in fura-2 fluorescence upon activation of AMPARs: Neuronal cultures loaded with fura-2 were imaged before, during, and after a 1 min exposure to 300  $\mu$ M kainate. After the kainate challenge, neurons were washed for 30 min in a physiological buffer either at pH 7.4 (A) or pH 6.2 (B). At the end of the washout period, neurons were exposed for 5 min to the cell permeant  $Zn^{2+}$  chelator TPEN (20  $\mu$ M). Traces show mean fura-2  $\Delta F$  (expressed as % over baseline of 340/380 nm ratios) ( $\pm$ SEM) of 13–24 neurons from one experiment representative of 5–6, respectively.

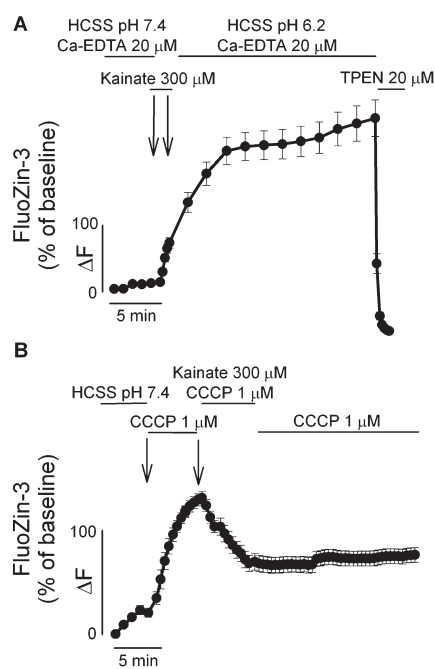
tion promotes a process of intracellular mobilization of  $Zn^{2+}$ , which is enhanced by low extracellular pH, chelation by  $Ca^{2+}$ -EDTA of any possible contaminant  $Zn^{2+}$  in the extracellular medium did not attenuate the FluoZin-3 fluorescent rises observed in the washout period (Figure 3A).

Mobilization of  $[Zn^{2+}]_i$  can occur from MT oxidation as well as from  $Zn^{2+}$  release from mitochondria (12,14,17). We first explored the possibility that AMPAR



**Figure 2.** Mild acidosis enhances AMPAR-mediated  $(Zn^{2+})_i$  rises. A, B. FluoZin-3 loaded cultures were exposed to kainate (300  $\mu$ M) for 1 min and washed out in a physiological buffer either at pH 7.4 (A) or pH 6.2 (B) for 20 min. Traces show mean FluoZin-3  $\Delta F$  ( $\pm$ SEM) of > 50 neurons from 4–7 experiments. C. Bar graph depicts the area under the curve during the recovery phase in the two conditions, \* indicates differences between washout at pH 7.4 versus washout at pH 6.2 ( $P < 0.01$ ). Note that AMPAR-mediated  $(Zn^{2+})_i$  rises are greatly enhanced upon exposure to an acidotic environment.

activation can induce release of  $Zn^{2+}$  present in mitochondria. In FluoZin-3 loaded cultures, exposure to a protonophore (CCCP; 1  $\mu$ M) that collapses the mitochondrial membrane potential and, as shown before (11–12) promotes  $Zn^{2+}$



**Figure 3.** AMPAR-mediated ( $Zn^{2+}$ )<sub>i</sub> rises are mainly dependent on intracellular  $Zn^{2+}$  mobilization. A. AMPAR-mediated ( $Zn^{2+}$ )<sub>i</sub> rises are not modulated by extracellular  $Zn^{2+}$  chelation. FluoZin-3 loaded neurons were exposed, as in Figure 1, to kainate in the presence of the extracellular  $Zn^{2+}$  chelator  $Ca^{2+}$  EDTA (20  $\mu$ M). Note that the ( $Zn^{2+}$ )<sub>i</sub> rises occurring both during and after kainate exposure are not affected by extracellular chelation of contaminant  $Zn^{2+}$ , indicating that the source of these rises is intracellular. B. AMPAR-mediated release of intra-mitochondrial  $Zn^{2+}$ . FluoZin-3 loaded cultures were exposed for 5 min to the mitochondrial protonophore, CCCP (1  $\mu$ M), challenged with 300  $\mu$ M kainate for 5 min and washed at pH 7.4, in the presence of CCCP. Note that prior exposure to CCCP greatly inhibits subsequent kainate-triggered ( $Zn^{2+}$ )<sub>i</sub> rises, indicating that these two manipulations target a common intracellular  $Zn^{2+}$  pool. Traces show time course of FluoZin-3  $\Delta F$  ( $\pm$ SEM), and are derived from > 15 neurons from 3–5 experiments.

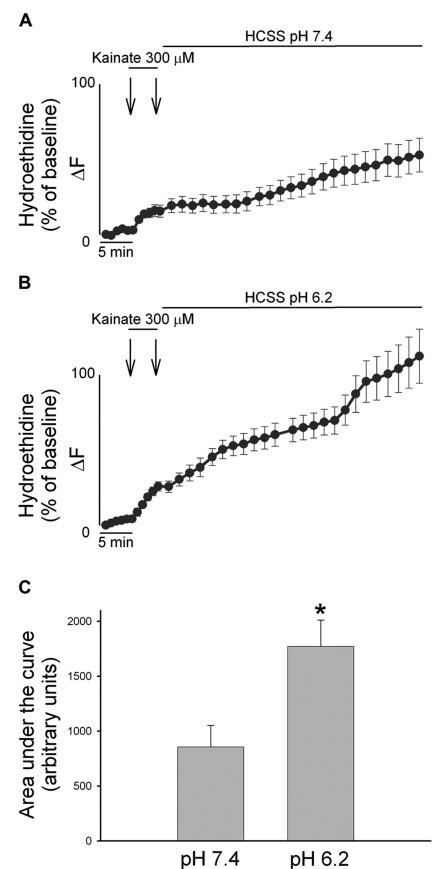
release from mitochondria, produced a large increase in cytosolic FluoZin-3 fluorescence. Suggesting that mitochondria are a likely source for AMPAR-mediated [ $Zn^{2+}$ ]<sub>i</sub> rises, CCCP exposure also oc-

cluded subsequent responses to kainate (Figure 3B).

On the contrary of NMDAR, AMPAR activation has been shown previously to generate very moderate oxidative stress of mitochondrial origin (21); therefore, a ROS-dependent  $Zn^{2+}$  mobilization seems less likely. We examined the possibility that AMPAR stimulation (at physiological and acidotic pH) can produce different levels of cytosolic ROS in neurons loaded with the superoxide sensitive fluorescent probe hydroethidine (HEt). Confirming the expected low oxidative stress produced by AMPAR activation under physiological conditions, Het-loaded neurons undergoing a 5 min kainate exposure showed very little increase in ROS production in the following 50 min when washed out in a buffer at pH 7.4 (Figure 4A). Interestingly, when the same excitotoxic challenge was followed by a 50 min wash at pH 6.2, ROS generation was found to be significantly higher (Figure 4B and C).

## DISCUSSION

Our present study complements previous  $Ca^{2+}$  imaging studies in both neuronal cultures and hippocampal slices and clearly indicates that excitotoxic activation of glutamate receptors leads not only to [ $Ca^{2+}$ ]<sub>i</sub> dyshomeostasis but also to a significant [ $Zn^{2+}$ ]<sub>i</sub> mobilization. This idea is substantiated by the interpretation of the increases in both fura-2 ratios and FluoZin-3 fluorescence (quenched by TPEN) that we observed. Fura-2 has a low affinity for  $Mg^{2+}$  ( $K_d$  6–10 mM) and exhibits an affinity for  $Zn^{2+}$  that is nearly 100-fold higher than its affinity for  $Ca^{2+}$  ( $K_d$  0.5–3 nM). Upon  $Zn^{2+}$  binding, fura-2 fluorescence undergoes changes similar to what is observed with  $Ca^{2+}$  (i.e. increases of the 340/380 nm excitation ratio). On the other hand, fura-2 binding to transition metals likely to be intracellularly released in significant amounts, such as manganese, ferrous and ferric iron, and copper results in different levels of quenching of the probe fluorescence.



**Figure 4.** AMPAR-mediated ROS generation is enhanced by low extracellular pH. A, B. Cortical cultures were loaded with HEt, and exposed for 5 min to 300  $\mu$ M kainate and washed for 50 min with a buffer at pH 7.4 (A) or pH 6.2 (B). HEt fluorescence changes for each neuron are expressed as the ratio of fluorescence at each time point ( $F_x$ ) to its own baseline fluorescence ( $F_0$ ). Traces show mean ( $\pm$  SEM) of 29 (A) or 40 (B) neurons from 3 experiments. C. Bar graph depicts the cumulative ROS production as area under the curve during the recovery phase in the two conditions, \* indicates differences between washout at pH 7.4 versus washout at pH 6.2 ( $P < 0.01$ ). Note the marked increase in HEt fluorescence only in the case of kainate exposures followed by an acidotic wash out.

FluoZin-3 is a  $Zn^{2+}$  selective indicator that exhibits high  $Zn^{2+}$  binding affinity ( $K_d$  for  $Zn^{2+}$   $\sim$ 15 nM), that is unperturbed by  $Ca^{2+}$  and  $Mg^{2+}$  concentrations up to at

least 0.1 mM and 1 M respectively (20). As with fura-2, iron and copper binding to FluoZin-3 produce quenching of the probe fluorescence. Thus, the only metal that could promote TPEN-sensitive increases in both fura-2 ratios and FluoZin-3 fluorescence is  $Zn^{2+}$ .

Our findings reinforce the idea that glutamate receptor overactivation can trigger a synergistic injurious interplay between  $Ca^{2+}$  and  $Zn^{2+}$ . Considering the fact that  $Zn^{2+}$  is able to induce neuronal loss with greater potency compared with  $Ca^{2+}$ , the cation might soon emerge as the major ionic determinant of excitotoxicity. Indeed, given the fact that significant  $[Zn^{2+}]_i$  release from mitochondria can result from large, NMDAR-triggered  $[Ca^{2+}]_i$  rises, and the likely probability that  $Ca^{2+}$ -induced mitochondrial ROS generation could also trigger  $Zn^{2+}$  mobilization from MTs, we might envision a pathological setting in which glutamate receptor-driven  $[Ca^{2+}]_i$  rises actually play a more modest injurious role than previously thought. Instead of being the executioner,  $[Ca^{2+}]_i$  dyshomeostasis can actually serve as an "accomplice" to deregulate the intracellular concentrations of the primary ionic mediator of neuronal injury,  $Zn^{2+}$ .

This study also integrates the main results of our previous experiments with the novel finding that reduction of extracellular pH to levels of parenchymal acidosis associated with cerebral ischemia (2) enhances AMPAR-mediated  $Zn^{2+}$  dyshomeostasis.

The connection between  $Zn^{2+}$  and acidosis is intriguing as pH reduction can affect many  $Zn^{2+}$  homeostatic systems. The permeability of major routes of  $Zn^{2+}$  entry, such as the VSCC and  $Ca^{2+}$ -permeable AMPAR, is enhanced at acidic pH, while the  $Ca^{2+}$  permeability of these channels (as well as NMDAR permeability) has been shown to be decreased under the same conditions (22–23).  $Zn^{2+}$  uptake and compartmentalization by Zinquin-positive cytosolic organelles is blocked by intracellular acidification, indicating the presence of a putative intracellular  $Zn^{2+}$ /proton

antiporter (24), and previous studies have also indicated that an acidotic environment can greatly destabilize  $Zn^{2+}$  binding to MTs and therefore promote  $[Zn^{2+}]_i$  accumulation (13). Interestingly,  $Zn^{2+}$  dyshomeostasis itself can alter the intracellular acid-base equilibrium and promote a feed-forward dyshomeostatic loop. Findings in cultured neurons suggest that  $Zn^{2+}$  can in fact favor intracellular acidification in a  $Ca^{2+}$ -dependent fashion through an increased activity of the  $Na^+/Ca^{2+}$  exchanger, and also maintain such acidification by  $Zn^{2+}$ -dependent inhibition of proton efflux via the  $Cl^-/HCO_3^-$  exchanger (25). Finally, as observed in our study, prolonged intracellular acidosis could further evoke  $[Zn^{2+}]_i$  dyshomeostasis by interfering with the overall neuronal redox state, either by reducing the activity of cellular antioxidant enzymes or increasing hydroxyl radical formation (26).

In our study, it appears that the acidotic/AMPAR-mediated  $[Zn^{2+}]_i$  rises observed in neuronal cultures can be attributed to combined mechanisms of mitochondrial release of the cation together with impaired buffering by MTs. The fact that mitochondrial depolarization prior to the kainate exposure completely occludes AMPAR-mediated  $[Zn^{2+}]_i$  increases seems to indicate that most of the  $Zn^{2+}$  is released from that subcellular compartment (Figure 3B). On the other hand, the impaired  $Zn^{2+}$  buffering under acidotic conditions that follows AMPAR activation appears to be related to a synergistic inhibition on  $Zn^{2+}$  binding to MTs that is promoted by protons either directly (13), or indirectly, by enhancing AMPAR-mediated ROS production. This process eventually leads to MT oxidation and further decreased  $Zn^{2+}$  buffering.

On a speculative note, our model, which emphasizes the role played by acidosis and AMPAR-mediated  $Zn^{2+}$  dyshomeostasis in excitotoxicity, might be relevant for the timing and selection of pharmacological intervention in neurological conditions associated with both acidosis and alteration of  $[Zn^{2+}]_i$  content

such as stroke, status epilepticus, and trauma. While hours away from the ischemic attack when the acid-base equilibrium is restored, NMDAR antagonists maintain a very important therapeutic value, AMPAR antagonists might result as a better option immediately after the insult given the fact that NMDARs are already blocked by post-ictal parenchymal acidosis (27). The importance of AMPAR antagonism has been demonstrated recently by an elegant series of studies in which blockade of the  $Ca^{2+}$ -permeable AMPARs has been found to be neuroprotective both in "in vitro" and "in vivo" models of cerebral ischemia (28–30). Work by our laboratory and the groups of Suzanne Zukin and Mike Bennett has convincingly demonstrated that, in the post-ischemic period, blockade of Ca-A/K channels by spermine derivatives can greatly inhibit neuronal death in the CA1 hippocampal region by counteracting both  $[Zn^{2+}]_i$  accumulation and  $Zn^{2+}$ -dependent injurious processes.

Targeting  $Zn^{2+}$  dyshomeostasis might also require further exploration, as recent animal studies involving the use of pyruvate to restore  $Zn^{2+}$ -dependent injurious depletion of cytosolic  $NAD^+$  levels showed a dramatic neuroprotective effect in the context of both global and focal cerebral ischemia (31–32).

A final technical issue raised by our findings concerns the need for highly selective  $Ca^{2+}$ -sensitive fluorescent probes. The warning contained in the paper by Grynkiewicz, Poenie and Tsien (33) indicating the high sensitivity of  $Ca^{2+}$ -sensitive fluorescent probes to heavy metals has been neglected. It is now clear that mobilization of intracellular  $Zn^{2+}$  can be a significant contributor to the fluorescence changes of  $Ca^{2+}$  fluorescent probes. This effect should no longer be ignored nor  $Ca^{2+}$  dependency claimed without a prior careful exclusion of  $Zn^{2+}$ -dependent phenomena.

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