# Measurement of Intracellular Free Zinc Concentrations Accompanying Zinc-Induced Neuronal Death

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Toxic zinc influx may contribute to selective neuronal death after transient global ischemia. We previously used the high-affinity ($K_D$ = 27 nM) fluorescent dye mag-fura-5 to detect initial increases in neuronal intracellular free $Zn^{2+}$ ($[Zn^{2+}]_i$) associated with brief $Zn^{2+}$ exposure. Here we used the specific low-affinity $Zn^{2+}$ indicator Newport Green ($K_D$ = 1 $\mu$M) to measure the peak levels of $[Zn^{2+}]_i$ attained during prolonged, toxic exposures to extracellular $Zn^{2+}$. Murine cortical cell cultures exposed for 5–10 min to 300 $\mu$M $Zn^{2+}$ in the presence of kainate or elevated extracellular $K^+$ developed widespread neuronal death over the next 24 hr. Such $Zn^{2+}$ exposure under depolarizing conditions was accompanied by a large increase in $[Zn^{2+}]_i$ reaching several hundred nanomolar, which gradually recovered over the next 20–40 min after termination of $Zn^{2+}$ exposure. Both the level of $[Zn^{2+}]_i$ elevation and the extent of subsequent neuronal death depended on the concentration of extracellular $Zn^{2+}$ between 30 $\mu$M and 1 mM. In contrast, exposure to 300 $\mu$M $Zn^{2+}$ in the presence of 300 $\mu$M NMDA resulted in little increase in $[Zn^{2+}]_i$ and little neuronal death, suggesting that NMDA receptor-gated channels are less important as a route of toxic $Zn^{2+}$ entry than voltage-gated calcium channels.

*Key words: voltage-gated calcium channels; calcium; depolarization; kainate; NMDA; neurotoxicity*

Zinc is the second most abundant transition metal in the body after iron. It is essential for normal cellular function (Vallee and Falchuk, 1993) and likely serves an additional signaling role in the CNS (Frederickson, 1989). The CNS contains high levels of chelatable $Zn^{2+}$ (Frederickson et al., 1983), which is largely localized into the synaptic vesicles of excitatory nerve terminals (Perez-Clausell and Danscher, 1985). On release, $Zn^{2+}$ alters the behavior of several ion channels and receptors (for review, see Harrison and Gibbons, 1994; Smart et al., 1994), including inhibition of NMDA receptors and potentiation of AMPA receptors (Peters et al., 1987; Westbrook and Mayer, 1987). $Zn^{2+}$ can be released from nerve terminals after transient global ischemia (Tonder et al., 1990), sustained seizures (Sloviter, 1985), and head trauma (Long et al., 1998), and conceivably may attain concentrations of several hundred micromolar (Assaf and Chung, 1984). Such elevations of $Zn^{2+}$ in the extracellular space may become neurotoxic (Choi et al., 1988), thus contributing to the pathogenesis of neuronal cell loss in these conditions (Koh et al., 1996; Choi and Koh, 1998, Long et al., 1998). A key first step in $Zn^{2+}$-induced neuronal death appears to be excessive influx across the plasma membrane, largely through voltage-gated $Ca^{2+}$ channels (Weiss et al., 1993).

To identify possible downstream mediators of zinc-induced cytotoxicity, it is important to determine the levels of intracellular free zinc ($[Zn^{2+}]_i$) attained after excessive zinc entry. Although the $Zn^{2+}$-selective fluorescent dye 6-methoxy-8-*p*-toluene sulfonamide quinoline has been used to locate pools of chelatable $Zn^{2+}$ (Frederickson et al., 1987; Weiss et al., 1993), its lipophilic nature and toxicity make it unsuitable for quantitative measurements of $[Zn^{2+}]_i$ in living cells. Two sulfonamide derivatives of quinoline, *N*-(6-methoxy-8-quinolyl)-*p*-carboxybenzoylsulfonamide (TFL) and Zinquin, have allowed quantitative measurement of $[Zn^{2+}]_i$ in hippocampal slices (Budde et al., 1997), thymocytes, pancreatic islet cells (Zalewski et al., 1993, 1994), and hepatocytes (Brand and Kleineke, 1996), but these load poorly into cultured neurons (Sensi et al., 1997). Recently, mag-fura-5 and mag-fura-2 have been used to measure changes in $[Zn^{2+}]_i$ after $K^+$-induced depolarization or glutamate receptor activation in cultured central neurons (Sensi et al., 1997; Cheng and Reynolds, 1998). Our previous study found initial increases in intracellular free $Zn^{2+}$ (reaching 10–30 nM) after brief (15 sec) exposures to extracellular $Zn^{2+}$ in combination with NMDA, kainate, or a depolarizing stimulus (Sensi et al., 1997). Cheng and Reynolds (1998) used longer periods of extracellular $Zn^{2+}$ application (5–10 min) and estimated that glutamate-stimulated $[Zn^{2+}]_i$ may have approached 100 nM. However, the known sensitivity of mag-fura-5 or mag-fura-2 to $Ca^{2+}$ or $Mg^{2+}$ was a major limitation in these studies, which had to use bathing solutions lacking these ions. In

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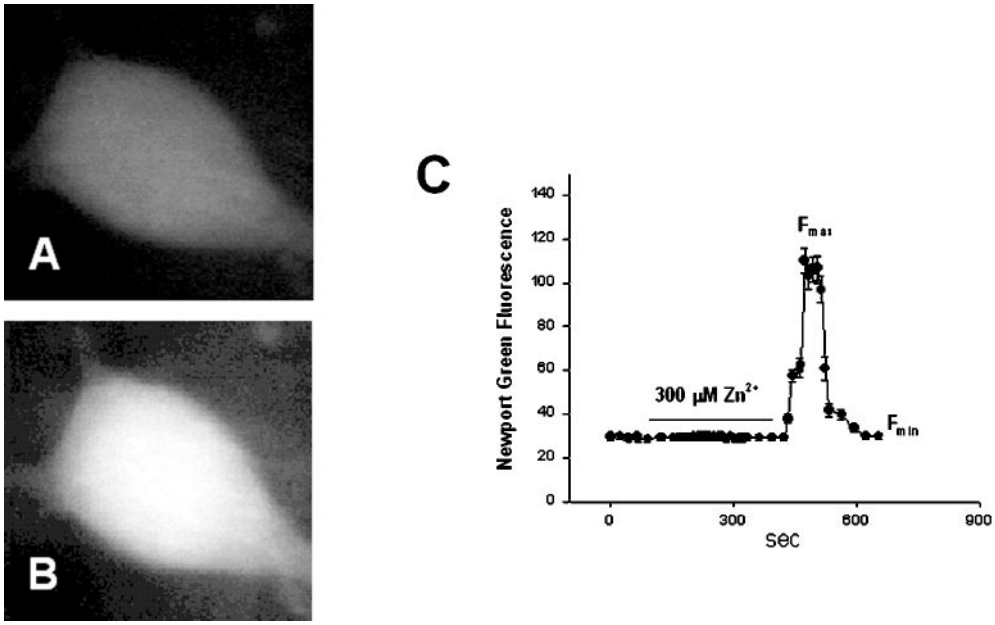


*Figure 1.* Newport Green detection of $[Zn^{2+}]_i$ in cortical neurons. *A*, *B*, Confocal image of a cortical neuron loaded with Newport Green diacetate before (*A*) and after (*B*) exposure to 1 mM $Zn^{2+}$ in the presence of 50 μM $Na^+$ pyrithione. *C*, Calibration of the fluorescent signal was performed at the end of each experiment. In this representative experiment, neurons were loaded with Newport Green and exposed for 5 min to 300 μM $Zn^{2+}$ in HCSS (1.8 mM $Ca^{2+}$, 0.8 mM $MgCl_2$). At the indicated times, 1 mM $Zn^{2+}$ + 50 μM $Na^+$-pyrithione or 100 μM TPEN were added to the bath.

addition, neither study used $Zn^{2+}$ exposures sufficient to induce neuronal death.

The purpose of the present study was to identify the level of neuronal $[Zn^{2+}]_i$ associated with lethal exposure to extracellular $Zn^{2+}$. Anticipating that this level might saturate fura-2 derivatives ($K_D$ for mag-fura-5 for $Zn^{2+}$ = 27 nM) (Sensi et al., 1997), we turned to the lower-affinity $Zn^{2+}$-selective fluorescent dye Newport Green ($K_D$ = 1 μM) (Haugland, 1996). Although nonratiometric, Newport Green has the additional desirable characteristic of being insensitive to $Ca^{2+}$ or $Mg^{2+}$, permitting measurements of $[Zn^{2+}]_i$ to be made in physiological solutions (Haugland, 1996).

Parts of this paper have been published previously in abstract form (Canzoniero et al., 1998).

## MATERIALS AND METHODS

*Cell culture.* Mixed cortical cultures, containing both neurons and glia, were prepared as described previously (Rose et al., 1993). Briefly, dissociated neocortices obtained from fetal mice at 14–16 d gestation were plated onto a previously established glial monolayer, at a density of 3–4 hemispheres per 24-well culture vessel (Falcon, Primaria, Franklin Lakes, NJ), in Eagle's minimal essential medium (MEM, Earle's salts) supplemented with 20 mM glucose, 2 mM glutamine, 5% fetal bovine serum, and 5% horse serum. Medium was changed after 1 week to MEM containing 20 mM glucose, 2 mM glutamine, and 10% horse serum, as well as cytosine arabinoside (final concentration 10 μM) to inhibit cell division. Subsequently, cultures were fed twice weekly with MEM supplemented with 20 mM glucose and 2 mM glutamine. Neurons for intracellular $Zn^{2+}$ imaging experiments were prepared similarly, using 35 mm glass-bottom dishes (MatTek, Ashland, MA) coated with poly-D-lysine/laminin (100:4 ng/ml). All experiments were performed between days 12 and 18 *in vitro.*

*Toxic exposure and assessment of injury.* Cultures were washed thoroughly before $Zn^{2+}$ exposure to remove all traces of serum. Brief exposures to $Zn^{2+}$ under depolarizing conditions (for 5 min) or in the presence of kainate or NMDA (for 10 min) were performed in HEPES-controlled salt solution (HCSS) containing (in mM): 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 20 HEPES, 5.5 glucose, 1.8 $CaCl_2$, 10 NaOH, pH 7.4, in room air at room temperature. High $K^+$ HCSS was prepared by substituting 60 mM KCl for an equimolar amount of NaCl. During the $Zn^{2+}$ exposure we attempted to limit the routes of $Zn^{2+}$ entry. Thus, NMDA exposures were performed in the presence of 6,7-dinitroquinoxaline-2,3-dione (NBQX, 10 μM), kainate exposures were performed in the presence of 10 μM MK-801, and KCl exposures were performed in the presence of both receptor antagonists. After exposure, the cultures were washed several times in MEM supplemented with 20 mM glucose and 10 μM NBQX (+10 μM MK-801 for NMDA conditions) to terminate the exposure and then returned to the incubator.

Overall neuronal death was assessed 24 hr after excitatory amino acid exposure, qualitatively by morphological observation using phase-contrast microscopy and quantitatively by measuring lactate dehydrogenase (LDH) efflux into the medium from damaged neurons. Previous experiments have demonstrated that the LDH release produced by glutamate exposure correlates well with the degree of neuronal loss determined by cell counts (Koh and Choi, 1987). A small amount of LDH was present in the media of cultures exposed to sham wash; this background release was determined in sister cultures for each experiment and subtracted from all values to yield the signal specifically associated with excitatory amino acid exposure. Each experiment also included a set of sister cultures exposed to 200 μM NMDA for 24 hr, a condition that induced virtually complete neuronal death without glial death.

*Intracellular $Zn^{2+}$ ($[Zn^{2+}]_i$) measurements.* To monitor $[Zn^{2+}]_i$, cell cultures were loaded with 5 μM Newport Green diacetate (excitation λ, 485 nm; emission λ, 530 nm) in the presence of 0.02% Pluronic F-127 for 30 min or at room temperature. Neurons were washed and incubated for an additional 30 min in the HCSS. After loading, neurons were washed twice with the same solution. All of the experiments were performed at room temperature under a constant perfusion (2 ml/min) on the stage of a Nikon Diaphot inverted microscope equipped with a 75 W Xenon lamp and a Nikon 40×, 1.3 N.A. epifluorescence oil immersion objective.

Images were acquired with a CCD camera (Quantex, Sunnyvale, CA) and digitized using Metafluor 2.5 software (Universal Imaging, West Chester, PA). Background fluorescence was subtracted at the beginning of each experiment.

*Confocal imaging.* Optical sections through cultured neurons loaded with Newport Green diacetate were imaged in z-series with a Noran-Odyssey laser confocal (Noran Instrument, Middleton, WI) with 488 nm excitation and >515 emission using a 60× oil objective with N.A. of 1.4 (Nikon). Images were collected with a confocal slit aperture of 15 μm and analyzed with an image analysis system (Metamorph, Universal Imaging).

*Materials.* Newport Green diacetate, 4-bromo-A 23187, tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN), and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). NBQX and MK-801 were purchased from Research Biochemicals (Natick, MA). $Na^+$-pyrithione, $ZnCl_2$, and other chemicals were obtained from Sigma (St. Louis, MO).

## RESULTS

Mixed cortical cultures containing neurons and glia were loaded with Newport Green as noted above. The addition of 1 mM $Zn^{2+}$

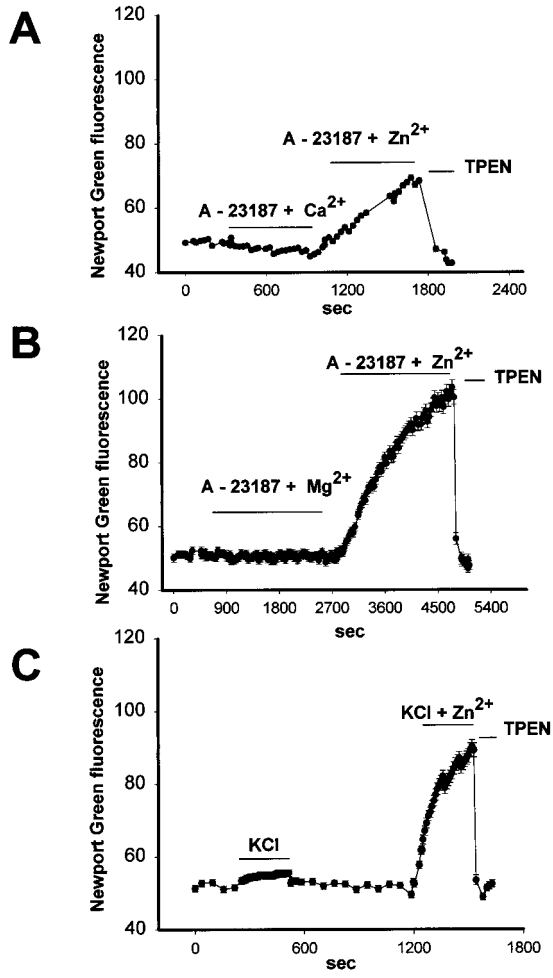


*Figure 2.* Specificity of Newport Green. *A*, A 10 min exposure to 10 $\mu$M 4-bromo-A-23187 in the presence of 10 mM $[Ca^{2+}]_o$ produced no change in Newport Green fluorescence. Subsequent exposure to 300 $\mu$M $Zn^{2+}$ in HCSS resulted in a progressive increase in fluorescence that returned to basal levels after the addition of 100 $\mu$M TPEN. *B*, A 30 min exposure to 10 $\mu$M 4-bromo-A-23187 + 30 mM in the presence of 30 mM $[Mg^{2+}]_o$ also produced no change in Newport Green fluorescence. Subsequent exposure to 300 $\mu$M $Zn^{2+}$ in HCSS resulted in a progressive increase in fluorescence that returned to the basal levels after the addition of 100 $\mu$M TPEN. *C*, A 5 min exposure to 60 mM KCl in HCSS produced no change in Newport Green fluorescence. Application of 60 mM KCl + 300 $\mu$M $Zn^{2+}$ resulted in a progressive increase in fluorescence that returned to the basal levels after the addition of 100 $\mu$M TPEN.

plus 50 $\mu$M Na-pyrithione [a zinc ionophore (Zalewski et al., 1993)] to the culture bathing medium was followed within 1–2 min with a sharp increase in Newport Green fluorescence. Examination of serial sections through neuronal cell bodies by confocal microscopy revealed a grossly uniform intracellular distribution to the dye fluorescence, both before and after addition of 1 mM $Zn^{2+}$ plus 50 $\mu$M $Na^+$-pyrithione (Fig. 1*A,B*).

$[Zn^{2+}]_i$ was calculated using *in situ* calibration at the end of each experiment. For each field of neurons a maximum fluorescence value ($F_{max}$) was obtained by adding 1 mM $Zn^{2+}$ plus 50 $\mu$M $Na^+$-pyrithione to the bathing medium, and a minimum fluorescence ($F_{min}$) was obtained by adding the membrane-permeable $Zn^{2+}$ chelator TPEN (100 $\mu$M) (Arslan et al., 1985) to a $Zn^{2+}$-, $Mg^{2+}$-, and $Ca^{2+}$-free bathing medium (Fig. 1*C*). A $K_D$ of 1 $\mu$M (Haugland, 1996) was assumed in the formula described by Grynkiewicz et al. (1985): $[Zn^{2+}]_i = K_D \cdot [(F - F_{min})/(F_{max} - F)]$. Calibration in solution using Newport Green dipotassium salt (cell-impermeable form, 0.5 $\mu$M) revealed that the dye permitted accurate measurements of $Zn^{2+}$ concentrations up to a few micromolar without evidence of saturation (data not shown).

Exposure to the divalent cation ionophore 4-bromo-A23187 (10 $\mu$M), together with either 10 mM $Ca^{2+}$ (10 min) or 30 mM $Mg^{2+}$ (30 min), did not result in a change in neuronal Newport Green fluorescence (Fig. 2*A,B*). However, the addition of 4-bromo-A23187 together with 300 $\mu$M $Zn^{2+}$ was followed by a sharp increase in dye fluorescence signal that was brought sharply back to basal levels by subsequent addition of the $Zn^{2+}$ chelator TPEN (100 $\mu$M) (Fig. 2*A,B*). Similarly, addition of 60 mM $K^+$ to the bathing medium (containing 1.8 mM $Ca^{2+}$and 0.8 mM $Mg^{2+}$) did not produce a change in dye fluorescence unless 300 $\mu$M $Zn^{2+}$ was added at the same time (Fig. 2*C*).

Neurons exposed to 60 mM $K^+$ plus 300 $\mu$M $Zn^{2+}$ exhibited an increase in $[Zn^{2+}]_i$, with partial recovery toward baseline after $Zn^{2+}$ removal (mean peak = 428 nM ± 176 SD, $n$ = 154 cells; five experiments) (Fig. 3*A,B*). The same $K^+$ plus $Zn^{2+}$ exposure was sufficient to produce widespread neuronal death 24 hr after exposure (Fig. 4*A*). Exposure to 300 $\mu$M kainate plus 300 $\mu$M $Zn^{2+}$ for 10 min also produced a large increase in neuronal $[Zn^{2+}]_i$ (mean peak = 401 nM ± 283 SD, $n$ = 183 cells; six experiments), with some cells exhibiting much higher values (>900 nM) (Fig. 3*C*). The same kainate plus $Zn^{2+}$ exposure induced widespread neuronal death 24 hr after exposure (Fig. 4*A*).

In contrast to the ability of high $K^+$ or kainate to produce marked increases in $[Zn^{2+}]_i$ during zinc exposures, exposure to 300 $\mu$M NMDA plus 300 $\mu$M $Zn^{2+}$ for 10 min resulted in a smaller increase in neuronal $[Zn^{2+}]_i$ (mean peak = 107 nM ± 69.5 SD, $n$ = 112 cells; three experiments) (Fig. 3*E,F*). The same NMDA plus $Zn^{2+}$ exposure produced little neuronal death 24 hr after exposure (Fig. 4*A*). To test the hypothesis that elevation in $[Zn^{2+}]_i$ was correlated with the amount of neuronal cell death observed in the above protocols, we tested the effect of varying extracellular $Zn^{2+}$ concentrations on $[Zn^{2+}]_i$ and cell death triggered by exposure to 60 mM KCl. Both peak $[Zn^{2+}]_i$ levels (Fig. 4*B*) and area under the curve (measured during and 10 min after $Zn^{2+}$ exposure) (data not shown) correlated with resultant neuronal death; the correlation was better with peak $[Zn^{2+}]_i$ levels.

## DISCUSSION

We used the newly developed, low-affinity $Zn^{2+}$-selective fluorescent dye Newport Green to measure $[Zn^{2+}]_i$ within cortical neurons exposed to cytotoxic concentrations of extracellular $Zn^{2+}$ in the presence of normal extracellular concentrations of $Ca^{2+}$ and $Mg^{2+}$. The main finding of our study is that peak levels of $[Zn^{2+}]_i$ induced with different stimuli and different concentrations of extracellular $Zn^{2+}$ correlated well with subsequent neuronal death, with little neuronal death occurring until peak $[Zn^{2+}]_i$ exceeded 250–300 nM. Consistent with other observations (Haugland, 1996), we found that Newport Green was not sensitive to even high concentrations of $Ca^{2+}$ or $Mg^{2+}$, administered together with the ionophore A23187. Other $Zn^{2+}$-sensitive dyes such as APTRA-BTC are sensitive to $Mg^{2+}$ (Haugland, 1996), and other $Zn^{2+}$-specific dyes, such as *N*-(6-methoxy-8-quinolyl)-*p*-carboxybenzoylsulfonamide (Budde et al., 1997) or Zinquin (Zalewski et al., 1994) appear for technical reasons to be less suitable than Newport Green for measuring $[Zn^{2+}]_i$ in cultured neurons (Sensi et al., 1997). Importantly, confocal microscopic examination of neuronal Newport Green

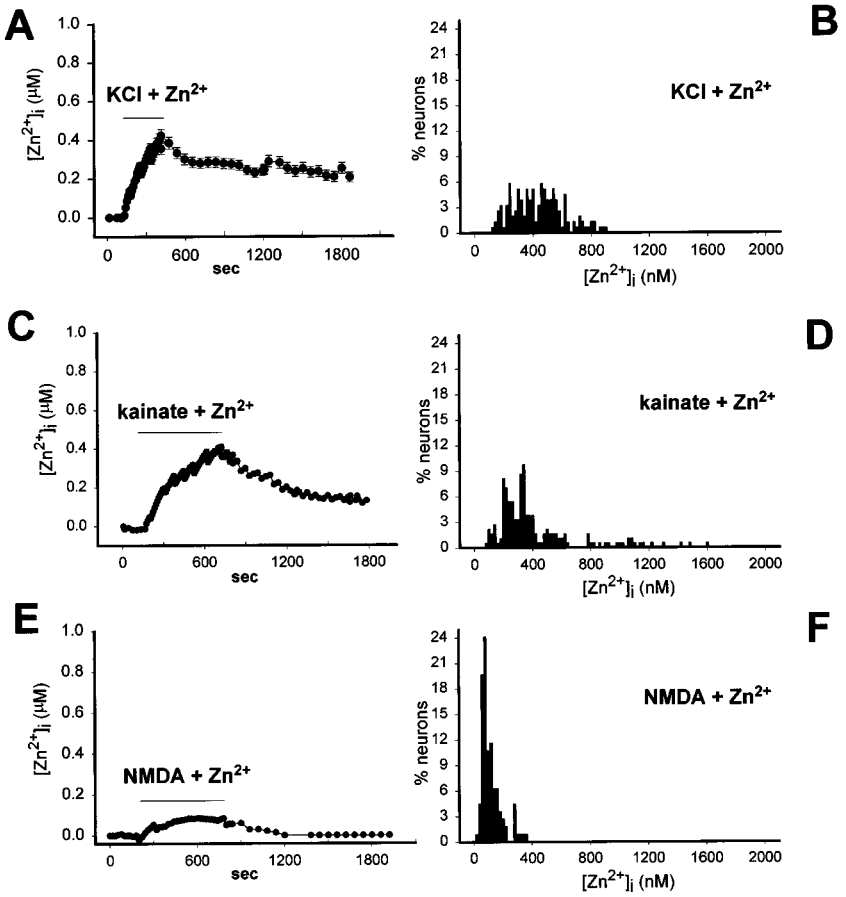


*Figure 3.* Changes in $[Zn^{2+}]_i$ induced by depolarization or glutamate agonist exposure. *A*, *B*, Cultures were exposed for 5 min to 60 mM KCl + 300 μM $Zn^{2+}$. MK-801 (10 μM) and NBQX (10 μM) were included during the exposure to block glutamate receptor activation by endogenous glutamate release. *A*, One representative field, $n$ = 40 neurons. *B*, Histogram plot of pooled data from five experiments (154 neurons), depicting the percentage of total neurons reaching each indicated peak $[Zn^{2+}]_i$ response. Each bin width was 20 nM $[Zn^{2+}]_i$. *C*, *D*, Cultures were exposed for 10 min to 300 μM kainate + 300 μM $Zn^{2+}$. MK-801 (10 μM) was included during the exposure to prevent NMDA receptor activation by endogenous glutamate release. *C*, One representative field, $n$ = 41 neuron. *D*, Histogram plot of pooled data from six experiments (183 neurons), depicting the percentage of total neurons reaching each indicated peak $[Zn^{2+}]_i$ response. Each bin width was 20 nM $[Zn^{2+}]_i$. *E*, *F*, Cultures were exposed for 10 min to 300 μM NMDA + 300 μM $Zn^{2+}$. NBQX (10 μM) was included during the exposure to prevent activation of AMPA or kainate receptors by endogenous glutamate release. *E*, One representative field, $n$ = 36 neurons. *F*, Histogram plot of pooled data from three experiments (112 neurons), depicting the percentage of total neurons reaching each indicated peak $[Zn^{2+}]_i$ response. Each bin width was 20 nM $[Zn^{2+}]_i$.

fluorescence did not reveal any gross inhomogeneities. Although it is likely that differences in local $[Zn^{2+}]_i$ concentrations exist within a given cell, reflecting subcellular domains such as lysosomes (Palmiter et al., 1996), the confocal imaging data suggest that it is reasonable to assign an average value to neuronal $[Zn^{2+}]_i$, in a manner similar to that typically done with $[Ca^{2+}]_i$.

Recent work using low-affinity $Ca^{2+}$ indicators has highlighted the technical limitations of using high-affinity dyes such as indo-1 and fura-2 to estimate levels of neuronal $[Ca^{2+}]_i$ achieved during $Ca^{2+}$overload protocols. Although fura-2 suggests that both lethal NMDA exposure and non-lethal AMPA exposure elevate cortical neuronal $[Ca^{2+}]_i$ to comparable levels (~300–400 nM), the low-affinity $Ca^{2+}$ indicator benzothiazole coumarin revealed that NMDA exposure resulted in severalfold higher $[Ca^{2+}]_i$ (Hyrc et al., 1997). Similarly, use of the low-affinity $Ca^{2+}$ indicator fura-2FF, but not fura-2, revealed that the GABAergic cortical neuronal subpopulation exhibiting preferential vulnerability to kainate toxicity developed markedly higher $[Ca^{2+}]_i$ levels in response to kainate exposure than the general neuronal population (Carriedo et al., 1998). Use of a high-affinity indicator (mag-fura-5, see above) to measure early changes in $[Zn^{2+}]_i$ in neurons exposed to $Zn^{2+}$ in the presence of NMDA, kainate, or high $K^+$ did not reveal differences among the different conditions. Cheng and Reynolds (1998) used another high-affinity $Zn^{2+}$ indicator, mag-fura-2 ($K_D$ = 20 nM), to measure $[Zn^{2+}]_i$ in forebrain neurons and observed comparable rises in neurons exposed to NMDA plus $Zn^{2+}$ versus neurons exposed to kainate plus $Zn^{2+}$. However, the known sensitivity of mag-fura-5 or mag-fura-2 to $Ca^{2+}$ or $Mg^{2+}$ was a major limitation in these studies, which had to use bathing solutions lacking these ions.

Here, use of the low-affinity $Zn^{2+}$ indicator Newport Green revealed that exposure to NMDA, which was relatively nontoxic, induced much lower increases in $[Zn^{2+}]_i$ than exposure to kainate or 60 mM $K^+$, which was relatively toxic. Although $Zn^{2+}$ may permeate through the NMDA receptor-gated channel (Mayer and Westbrook, 1987; Ascher and Novak 1988; Christine and Choi 1990; Koh and Choi, 1994; Sensi et al., 1997), this route is likely less important than voltage-gated neuronal $Ca^{2+}$ channels (Weiss et al., 1993). In addition, $Zn^{2+}$ itself attenuates the opening of NMDA receptor-gated channels by both voltage-dependent and voltage-independent mechanisms (Peters et al., 1987; Westbrook and Mayer, 1987); 300 μM extracellular $Zn^{2+}$ is likely to inhibit NMDA receptor activation. A good correlation between attained peak levels of $[Zn^{2+}]_i$ and subsequent neuronal death was also observed when cultures were exposed to 60 mM $K^+$ and varying concentrations of extracellular $Zn^{2+}$.

An especially wide range of $[Zn^{2+}]_i$ levels were seen after kainate stimulation, consistent with the existence of two different neuronal cell populations: a general population and a subpopulation expressing $Ca^{2+}$- and $Zn^{2+}$-permeable AMPA/kainate receptors (Turetsky et al., 1994; Yin and Weiss, 1995; Sensi et al., 1997). As this manuscript was being prepared, Sensi et al. (1999) reported the use of Newport Green to demonstrate specifically that the cultured cortical neuronal subpopulation expressing $Ca^{2+}$- and $Zn^{2+}$-permeable AMPA/kainate receptors [identified by kainate-stimulated $Co^{2+}$ uptake; Pruss et al. (1991)] indeed responded to challenge with kainate plus $Zn^{2+}$ with higher levels of $[Zn^{2+}]_i$ than did the general cortical neuronal population.

Little is known about intracellular $Zn^{2+}$ homeostasis, but

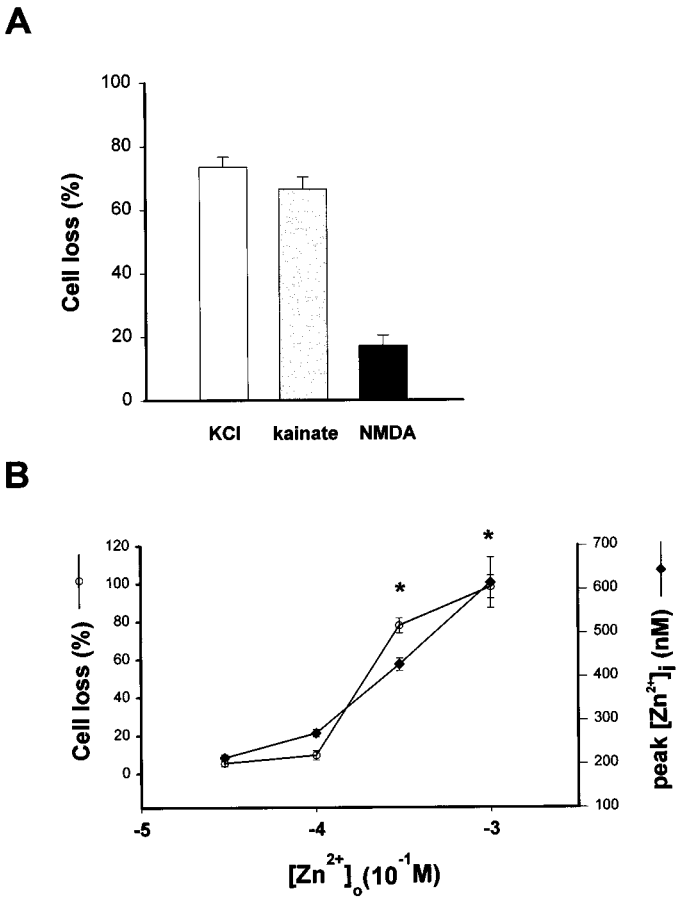


*Figure 4.* Zinc-induced neuronal death. *A*, Neuronal cell death resulting from exposure to $Zn^{2+}$ in the presence of depolarizing conditions or glutamate agonists. Cultures exposed to KCl/$Zn^{2+}$ (60 mM KCl, 300 $\mu$M $Zn^{2+}$, 10 $\mu$M MK-801, 10 $\mu$M NBQX, for 5 min) or kainate/$Zn^{2+}$ (300 $\mu$M kainate, 300 $\mu$M $Zn^{2+}$, 10 $\mu$M MK-801, for 10 min) resulted in the death of the majority of cultured cortical neurons, as assessed by LDH release 24 hr later. However, exposure to NMDA/$Zn^{2+}$ (300 $\mu$M NMDA, 300 $\mu$M $Zn^{2+}$, 10 $\mu$M NBQX, for 10 min) caused little cell loss. Values represent mean (±SEM) levels of cell loss determined by LDH release ($n$ = 5–10 different experiments per value). *B*, Depolarization-induced changes in peak $[Zn^{2+}]_i$ and resultant neuronal death depend on extracellular $Zn^{2+}$ concentration. Cultures were exposed for 5 min to 60 mM KCl at the indicated concentrations of extracellular $Zn^{2+}$ between 30 $\mu$M and 1 mM. Values represent peak (± SEM) $[Zn^{2+}]_i$ (obtained from $n$ = 3–5 different experiments) or mean (±SEM) levels of cell loss (determined by LDH release, $n$ = 5–10 different experiments for each value). * indicates difference from sham wash at $p < 0.05$ (two-way ANOVA followed by Student's Newman–Keuls test).

resting neuronal levels of $[Zn^{2+}]_i$ may be quite low, because metallothioneins have very high $Zn^{2+}$ affinity [$K_D$ for $Zn^{2+}$ $\sim 10^{-13}$ M (Maret, 1994)]. In the present study using Newport Green, or in previous study using mag-fura-5 (Sensi et al., 1997), we did not detect any baseline TPEN-sensitive fluorescence; of note, rodent brains develop synaptic $Zn^{2+}$ stores only during postnatal development (Slomianka and Geneser, 1997). In human erythrocytes, the behavior of anion-dependent $Zn^{2+}$ transport has suggested $[Zn^{2+}]_i$ levels below 3 nM (Kalfakakou and Simons, 1990). On the other hand, use of the fluorescent dye Zinquin to measure $[Zn^{2+}]_i$ has revealed levels of 0.6–2.7 $\mu$M in hepatocytes (Brand and Kleineke, 1996) and >10 $\mu$M in lymphoid cells (Zalewski et al., 1993). Possibly, hepatocytes regulate $[Zn^{2+}]_i$ differently compared with cortical neurons. Alternatively, these very high $[Zn^{2+}]_i$ levels may reflect uneven intracellular distribution of Zinquin (Zalewski et al., 1993; Reyes, 1996; Sensi et al., 1997).

$Zn^{2+}$ interacts with many proteins and other macromolecules (O'Halloran, 1993; Vallee and Falchuk, 1993), so near-micromolar elevations in $[Zn^{2+}]_i$ may produce injurious derangements in many cellular processes. For example, $Zn^{2+}$ can alter the behavior of protein kinase C (Hedberg et al., 1994), transcription factors such as NF-$\kappa$B (Shumilla et al., 1998), $Ca^{2+}$ binding proteins such as calmodulin and S100B (Baudier et al., 1983), and mitochondrial respiratory enzymes (Brand and Soling, 1986; Krotkiewska and Banas, 1992; Link and von Jagow, 1995). Present data may help narrow the search for key downstream mediators of zinc toxicity by providing quantitative constraints. In particular, present data strengthen the hypothesis that an important general contributing factor to zinc-induced neuronal death may be inhibition of glycolysis. Concentrations of $Zn^{2+}$ (100–200 nM) can inhibit fructose 1,6-diphosphatase ($IC_{50}$ = 100 nM) and glyceraldehyde 3-phosphate dehydrogenase ($IC_{50}$ = 150 nM) (Maret et al., 1999). Administration of the downstream energy substrate pyruvate can attenuate both $Zn^{2+}$-induced depletion of neuronal ATP levels and $Zn^{2+}$-induced neuronal death (Sheline and Choi, 1997). In the cortical neuronal subpopulation expressing $Ca^{2+}$- and $Zn^{2+}$-permeable AMPA/kainate receptors, where $[Zn^{2+}]_i$ after toxic $Zn^{2+}$ exposure may exceed 1 $\mu$M, selective potentiation of mitochondrial free radical generation may contribute to neuronal death (Sensi et al., 1999).

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