The mammalian CNS contains an abundance of chelatable \( \text{Zn}^{2+} \) sequestered in the vesicles of glutamatergic terminals. These vesicles are particularly numerous in hippocampal mossy fiber synapses of the hilar and CA3 regions (Haug, 1967; Perez-Clausell and Danscher, 1985; Frederickson, 1989; Frederickson et al., 2000; Li et al., 2001). A possible synaptic signaling role for \( \text{Zn}^{2+} \) is suggested by its interactions with excitatory and inhibitory amino acid receptors such as NMDA, AMPA, and GABA receptors (Peters et al., 1987; Westbrook and Mayer, 1987). \( \text{Zn}^{2+} \) accumulates in synaptic vesicles through a specific \( \text{Zn}^{2+} \) transporter, termed \( \text{Zn} \) transporter 3 (Palmieri et al., 1996). Vesicular \( \text{Zn}^{2+} \) release can be elicited by electrical stimulation (Howell et al., 1984; Li et al., 2001) or membrane depolarization (by elevating extracellular K\(^+\) concentration; Assaf and Chung, 1984; Aniksztejn et al., 1987; Li et al., 2001). Characterization of this \( \text{Zn}^{2+} \) release has revealed that it is released in the same manner as neurotransmitters: the release is \( \text{Ca}^{2+} \)-dependent and tetrodotoxin-sensitive (Li et al., 2001). Recently, it has been shown that extracellular \( \text{Zn}^{2+} \) enters neurons through glutamate receptors and voltage-dependent \( \text{Ca}^{2+} \) channels (VDCCs; Weiss and Sensi, 2000). Despite a considerable amount of evidence suggesting that \( \text{Zn}^{2+} \) acts in concert with neurotransmitters in the CNS, a specific physiological role for synaptically released \( \text{Zn}^{2+} \) has yet to be identified.

In addition to having routes of entry into neurons that are activated during nerve transmission, \( \text{Zn}^{2+} \) is known to interact with the protein kinases and phosphatases of signal transduction pathways that affect changes in gene expression (Brewer et al., 1979; Hubbard et al., 1991; Weinberger and Rostas, 1991; Quest et al., 1992; Maret et al., 1999; Park and Koh, 1999; Lengyel et al., 2000). Our recent observation (Li et al., 2001) of frequency-dependent \( \text{Zn}^{2+} \) release from mossy fiber synaptic terminals and subsequent entry into postsynaptic neurons of the dentate gyrus has suggested to us that \( \text{Zn}^{2+} \) might play a role in the normal physiological function of these neurons. Detectable \( \text{Zn}^{2+} \) release varied over a range of frequencies (10–200 Hz), which included frequencies used to induce long-term potentiation (LTP). We hypothesized that translocation of \( \text{Zn}^{2+} \) across synapses might be an important physiological signal mediating some aspects of synaptic plasticity, such as LTP.

LTP is an important model for studying the cellular mechanisms of neuronal plasticity, learning, and memory. \( \text{Zn}^{2+} \)-deficient rats and rhesus monkeys experience a learning and working memory deficit (Golub et al., 1995). Although the possibility that \( \text{Zn}^{2+} \) released from the mossy fiber bouton might be involved in hippocampal LTP has been proposed by Weiss et al (1989) more than a decade ago, much is still unknown about the involvement of synaptically released \( \text{Zn}^{2+} \) in synaptic plasticity. Recently, two groups failed to alter the induction of mossy fiber LTP by removing synaptically released \( \text{Zn}^{2+} \) with the \( \text{Zn}^{2+} \) chelator CaEDTA (Lu et al., 2000; Vogt et al., 2000). Using a long-lasting potentiation of synaptic transmission that lasted more than 3 hr. Moreover, our experiments indicate the effects of \( \text{Zn}^{2+} \) were not attributable to its interaction with extracellular membrane proteins but required its entry into presynaptic or postsynaptic neurons. Co-released glutamate is also essential for induction of LTP under physiological conditions, in part because it allows \( \text{Zn}^{2+} \) entry into postsynaptic neurons. These results indicate that synaptically released \( \text{Zn}^{2+} \), acting as a second messenger, is necessary for the induction of LTP at mossy fiber→CA3 synapses of hippocampus.

Key words: zinc; long-term potentiation; CA3; hippocampus; CaEDTA; mossy fiber; plasticity; Na-pyritone; Newport Green; synaptic transmission.

The mammalian CNS contains an abundance of chelatable \( \text{Zn}^{2+} \) sequestered in the vesicles of glutamatergic terminals. These vesicles are particularly numerous in hippocampal mossy fiber synapses of the hilar and CA3 regions (Haug, 1967; Perez-Clausell and Danscher, 1985; Frederickson, 1989; Frederickson et al., 2000; Li et al., 2001). A possible synaptic signaling role for \( \text{Zn}^{2+} \) is suggested by its interactions with excitatory and inhibitory amino acid receptors such as NMDA, AMPA, and GABA receptors (Peters et al., 1987; Westbrook and Mayer, 1987). \( \text{Zn}^{2+} \) accumulates in synaptic vesicles through a specific \( \text{Zn}^{2+} \) transporter, termed \( \text{Zn} \) transporter 3 (Palmieri et al., 1996). Vesicular \( \text{Zn}^{2+} \) release can be elicited by electrical stimulation (Howell et al., 1984; Li et al., 2001) or membrane depolarization (by elevating extracellular K\(^+\) concentration; Assaf and Chung, 1984; Aniksztejn et al., 1987; Li et al., 2001). Characterization of this \( \text{Zn}^{2+} \) release has revealed that it is released in the same manner as neurotransmitters: the release is \( \text{Ca}^{2+} \)-dependent and tetrodotoxin-sensitive (Li et al., 2001). Recently, it has been shown that extracellular \( \text{Zn}^{2+} \) enters neurons through glutamate receptors and voltage-dependent \( \text{Ca}^{2+} \) channels (VDCCs; Weiss and Sensi, 2000). Despite a considerable amount of evidence suggesting that \( \text{Zn}^{2+} \) acts in concert with neurotransmitters in the CNS, a specific physiological role for synaptically released \( \text{Zn}^{2+} \) has yet to be identified.

In addition to having routes of entry into neurons that are activated during nerve transmission, \( \text{Zn}^{2+} \) is known to interact with the protein kinases and phosphatases of signal transduction pathways that affect changes in gene expression (Brewer et al., 1979; Hubbard et al., 1991; Weinberger and Rostas, 1991; Quest et al., 1992; Maret et al., 1999; Park and Koh, 1999; Lengyel et al., 2000). Our recent observation (Li et al., 2001) of frequency-dependent \( \text{Zn}^{2+} \) release from mossy fiber synaptic terminals and subsequent entry into postsynaptic neurons of the dentate gyrus has suggested to us that \( \text{Zn}^{2+} \) might play a role in the normal physiological function of these neurons. Detectable \( \text{Zn}^{2+} \) release varied over a range of frequencies (10–200 Hz), which included frequencies used to induce long-term potentiation (LTP). We hypothesized that translocation of \( \text{Zn}^{2+} \) across synapses might be an important physiological signal mediating some aspects of synaptic plasticity, such as LTP.

LTP is an important model for studying the cellular mechanisms of neuronal plasticity, learning, and memory. \( \text{Zn}^{2+} \)-deficient rats and rhesus monkeys experience a learning and working memory deficit (Golub et al., 1995). Although the possibility that \( \text{Zn}^{2+} \) released from the mossy fiber bouton might be involved in hippocampal LTP has been proposed by Weiss et al (1989) more than a decade ago, much is still unknown about the involvement of synaptically released \( \text{Zn}^{2+} \) in synaptic plasticity. Recently, two groups failed to alter the induction of mossy fiber LTP by removing synaptically released \( \text{Zn}^{2+} \) with the \( \text{Zn}^{2+} \) chelator CaEDTA (Lu et al., 2000; Vogt et al., 2000). Using a long-lasting potentiation of synaptic transmission that lasted more than 3 hr. Moreover, our experiments indicate the effects of \( \text{Zn}^{2+} \) were not attributable to its interaction with extracellular membrane proteins but required its entry into presynaptic or postsynaptic neurons. Co-released glutamate is also essential for induction of LTP under physiological conditions, in part because it allows \( \text{Zn}^{2+} \) entry into postsynaptic neurons. These results indicate that synaptically released \( \text{Zn}^{2+} \), acting as a second messenger, is necessary for the induction of LTP at mossy fiber→CA3 synapses of hippocampus.

Key words: zinc; long-term potentiation; CA3; hippocampus; CaEDTA; mossy fiber; plasticity; Na-pyritone; Newport Green; synaptic transmission.
fluorescence imaging, we show here that although a low concentration (1 mM) of CaEDTA was not sufficient to prevent synaptically released Zn²⁺ from reaching postsynaptic neurons after high-frequency stimulation (HFS), a higher concentration (10 mM) of Zn²⁺ chelator was. This treatment blocked the induction of LTP. Moreover, perfusion of slices with exogenous Zn²⁺ (50–100 µM) could also induce long-lasting potentiation of the EPSP in the absence of HFS. Finally, our experiments indicate that the effects of Zn²⁺ were not attributable to its interaction with extracellular membrane proteins but required its entry into presynaptic or postsynaptic neurons.

**MATERIALS AND METHODS**

**Hippocampal slice preparation.** Experiments were conducted according to the principles set forth in the Guide for Care and Use of Laboratory Animals (Institute of Animal Resources, National Research Council, National Institutes of Health publication 74-23). Male adult Sprague Dawley rats were anesthetized with ketamine hydrochloride and decapitated. The brain was quickly removed and immersed in ice-cold (1–4°C) artificial CSF (ACSF) with the composition of (in mM): NaCl, 124; KCl, 1.75; MgSO₄, 1.3; CaCl₂, 2.4; KH₂PO₄, 1.25; NaHCO₃, 26; and dextrose, 10, continuously bubbled with 95% O₂ and 5% CO₂. Transverse hippocampal slices 400–450 µm in thickness were prepared using a McIlwain tissue chopper (Brinkmann Instruments, Westbury, NY) or Vibratome (Frederic Haer, Brunswick, ME). Slices were incubated in a 95% O₂-5% CO₂ atmosphere containing 0.1% pluronic acid for 1 hr and then washed with ACSF. The Zn²⁺ could also induce long-lasting potentiation of the EPSP in the absence of HFS. Finally, our experiments indicate that the removal of Zn²⁺ after HFS did not influence the late, or maintenance, phase of LTP.

**RESULTS**

**Rapid chelation of synaptically released Zn²⁺ blocks induction of mossy fiber LTP.** To determine whether Zn²⁺ is involved in this specific form of synaptic plasticity, we examined the effects of applying CaEDTA, a cell-impermeable extracellular Zn²⁺ chelator (K₅ = 10⁻¹⁶.4 M) that does not appreciably alter Ca²⁺ concentration (Fredens and Danscher, 1973; Dawson et al., 1986; Bers et al., 1994). Stimulation of the mossy fiber axons produced an extracellular field EPSP recorded in the dendritic region (stratum lucidum) of pyramidal neurons in field CA3 of hippocampal slices (Fig. 1A). In control slices, brief HFS produced EPSP potentiation (mossy fiber LTP, Fig. 1B). The averaged normalized EPSP slope 30 min after HFS was 193 ± 10% (mean ± SEM; n = 9) of baseline, and the potentiation was stable for >3 hr (maximum recording duration) after our standard recording procedure (Bramham and Sur, 1996). To chelate Zn²⁺ released by HFS and to prevent it from reaching postsynaptic neurons, we perfused slices with 10 mM CaEDTA for 10 min before and 10 min after HFS. This treatment blocked induction of LTP (103 ± 4%, mean ± SEM; n = 11; Fig. 1C). Although there was an initial small post-tetanic potentiation immediately after HFS, it decayed to baseline within 5–10 min. These results suggest that Zn²⁺ released during HFS plays an essential role in the induction of mossy fiber LTP. To determine whether chelation of Zn²⁺ could alter the maintenance of LTP, CaEDTA was applied 10 min after HFS. Figure 1D shows that CaEDTA (10 mM) had no effect on established LTP, suggesting that the removal of Zn²⁺ after HFS did not influence the late, or maintenance, phase of LTP.

**Kinetics of Zn²⁺ chelation by CaEDTA**

To verify that Zn²⁺ released from mossy fiber terminals by HFS was adequately chelated by 10 mM CaEDTA, we loaded slices with the selective extracellular Zn²⁺ fluorescent indicator Newport Green dipotassium salt (cell-impermeable; Molecular Probes) and visualized Zn²⁺ release after stimulation in the presence and absence of CaEDTA. In a previous study (Li et al., 2001), we found that electrically stimulated Zn²⁺ release was frequency-dependent and could be detected with as little as 0.5 Hz stimulation at 500 µA. The degree of Zn²⁺ release also increased with increasing stimulus amplitudes ranging from 20 to 500 µA (100 Hz over 5 sec). Ten millimolar CaEDTA chelated 85% of the synaptically released Zn²⁺, as indicated by Newport Green fluorescence (Fig. 2A). This result verifies that the effects of CaEDTA on LTP induction in mossy fiber–CA3 synapses was achieved by its selective chelation of the synaptically released Zn²⁺ from mossy fiber terminals. After bath perfusion of 10 mM CaEDTA for 10 min, followed by washout of the CaEDTA, we could still induce normal release of Zn²⁺ in normal ACSF (data not shown). On the other hand, a lower concentration of CaEDTA (1 mM) failed to reduce the extracellular Zn²⁺ after HFS, as evidenced by its inability to reduce Newport Green fluorescence (Fig. 2A). Therefore, induction of LTP was essentially unaffected in 1 mM CaEDTA (Fig. 2B).

These results are consistent with our calculations of the kinetics of Zn²⁺ chelation in ACSF containing CaEDTA. Our calcula-
Mossy Fiber—CA3 LTP Requires Translocation of Released Zn²⁺

Figure 1. Rapid chelation of extracellular Zn²⁺ blocks induction of mossy fiber LTP. A, Schematic of a hippocampal slice showing stimulating and recording sites. The traces on the right show the field EPSPs evoked by stimulation of mossy fibers (mf) and the commissural–associational pathway. The group II mGluR agonist DCG-IV (5 μM) selectively blocked mossy fiber responses to 17.3 ± 2% (n = 6) of those before drug application but had little effect on the responses (to 97.7 ± 1.9%; n = 6) evoked by stimulating the commissural–associational fibers in the stratum radiatum (electrode not shown). B, Mossy fiber LTP (193 ± 10%; n = 9) induced by HFS after recording a 30 min baseline (see Materials and Methods). C, HFS failed to induce mossy fiber LTP (103 ± 4%; n = 11) in the presence of 10 mM CaEDTA (dashed line). D, Adding CaEDTA (10 mM; dashed line) 10 min after induction of LTP did not affect the late, or maintenance, phase of LTP (175 ± 8%; n = 3). Each point in B–D represents the averaged and normalized EPSP initial negative slope, and error bars indicate SEM. Arrows indicate HFS (100 Hz, 2 sec). B–D, Insets, EPSP recorded during baseline and at the end of the recording period after HFS. Calibration: 1.0 mV, 5 msec.

Removal of synaptically released Zn²⁺ does not affect basal synaptic transmission

Disodium EDTA nominally saturated with equimolar Ca²⁺ (CaEDTA) has been used to add the chelator to physiological buffers such as ACSF without appreciably reducing the concentration of extracellular Ca²⁺, which is essential for normal synaptic transmission. To verify this, we measured the concentration of free Ca²⁺ in ACSF using a Ca²⁺ electrode. Addition of 1 mM CaEDTA did not alter the free Ca²⁺. Addition of 10 mM CaEDTA reduced the measured concentration of free Ca²⁺ from 2.25 ± 0.02 mM (mean ± SEM; n = 3) to 2.03 ± 0.03 mM (mean ± SEM; n = 3; Fig. 4A). This was probably attributable to incomplete saturation of the EDTA with Ca²⁺ during its manufacture. When we added an extra 0.22 mM CaCl₂ to ACSF containing 10 mM CaEDTA to compensate for the Ca²⁺ deficit, the concentration of free Ca²⁺ was 2.25 ± 0.01 mM (mean ± SEM; n = 3).

Ten millimolar CaEDTA had no effect on basal synaptic transmission elicited by low-frequency stimulation, as measured by the initial slope of the EPSP at mossy fiber→CA3 synapses (Fig. 4B). The chelator at this concentration also had no effect on paired pulse facilitation, a physiological property of presynaptic terminal function at mossy fiber→CA3 synapses (Fig. 4C). Our data demonstrate that basal synaptic function is not altered in the presence of 10 mM Zn²⁺ chelator. Thus, the data suggest that the effects of CaEDTA we observed were attributable to changes in Zn²⁺ concentration.

Mimicking Zn²⁺ release by adding exogenous Zn²⁺ induces a long-lasting potentiation of synaptic transmission

In the experiments described above, 10 mM CaEDTA apparently blocked the induction of LTP, because it chelated the Zn²⁺ released during HFS. We then hypothesized that the addition of exogenous Zn²⁺ to extracellular bathing solution in the absence of HFS would enhance the strength of synaptic transmission and would induce a long-lasting potentiation of the EPSP in field CA3. As shown in Figure 5A, the EPSP was gradually potentiated to 195 ± 17% of baseline (mean ± SEM; n = 8) during a 20 min exposure to 100 μM Zn²⁺ and remained potentiated for >3 hr. Zn²⁺ does not affect the afferent volley in our experimental conditions predict that, in 10 mM CaEDTA, 100 μM extracellular Zn²⁺ would be reduced to 33 nM within 0.1 msec, whereas in 1 mM CaEDTA, this concentration of Zn²⁺ would only be reduced to 15 μM in 0.1 msec (Fig. 3; and Appendix). Thus, we suggest that 10 mM CaEDTA effectively removes Zn²⁺ from nerve terminals by HFS before its physiological function can be performed (Basolo and Pearson, 1967; Davis et al., 1999). This explains why 1 mM CaEDTA failed to block induction of LTP in this study (Fig. 2B) and in previous studies (Lu et al., 2000; Vogt et al., 2000).
conditions. Throughout this experiment, the only stimulation applied was low-frequency test stimuli. These data strongly suggest that Zn²⁺/H₁₁₀₀₁ is able to enhance synaptic strength at mossy fiber CA3 synapses. Once long-lasting potentiation of the EPSP was established, washing away the exogenous Zn²⁺/H₁₁₀₀₁ with ACSF containing 10 mM CaEDTA (10 min) did not halt the potentiation (Fig. 5A). This result also indicates that the potentiating effect of Zn²⁺/H₁₁₀₀₁ was not attributable to its prolonged binding to plasma membrane components. Figure 5B shows the frequency (given as a percentage) with which various concentrations of Zn²⁺/H₁₁₀₀₁ induced long-lasting potentiation. In these concentration–response studies, we could reliably induce long-lasting potentiation of the EPSP in concentrations of 50 μM (71%; n = 7) and 100 μM (100%; n = 9) Zn²⁺/H₁₁₀₀₁. Estimates of the concentration of Zn²⁺/H₁₁₀₀₁ released from the mossy fibers during HFS have ranged from 10 to 100 μM (Vogt et al., 2000; Li et al., 2001); Zn²⁺ concentration could reach as much as 300 μM under extreme conditions (Frederickson, 1989). In the presence of 300 μM Zn²⁺/H₁₁₀₀₁, we observed long-lasting potentiation of the EPSP in less than half the slices tested (n = 7; Fig. 5B). This high failure rate might be caused by the neurotoxic effect of Zn²⁺ at this high concentration (Frederickson et al., 2000).

**Figure 2.** Zn²⁺ release with electrical stimulation and the effects of Zn²⁺ chelator. A. Extracellular Zn²⁺ released from terminals after electrical stimulation (stim.) was measured as the peak emission intensity of extracellular Newport Green fluorescence in the absence and presence of Zn²⁺ chelator (3 determinations; error bars indicate SE). The paired images on top correspond with each condition of the bar graph. They were acquired before (controls in the first row) and after (second row) HFS (200 μsec, 0.5 mA pulses for 5 sec). In these false-color images, increasing intensity of Zn²⁺ fluorescence is represented by the spectrum ranging from blue (the lowest) to red (the highest). Scale bar, 100 μm. A’. The hatched area represents the region where images were acquired. H, Hilus. Values plotted are the mean ± SEM; n = 4; *p < 0.05. A”, Plot of electrical stimulation (100 Hz for 5 sec)-evoked rapid release of Zn²⁺ from neuronal terminals measured by changes in Newport Green fluorescence intensity (arbitrary units). The arrow indicates the beginning of stimulation. B, A low concentration of CaEDTA (1 mm; dashed line) had little effect on mossy fiber LTP (227 ± 11%; n = 4) evoked by HFS. Each point is the averaged and normalized EPSP initial negative slope, and error bars indicate SEM. Arrows indicate the time giving HFS (100 Hz, 2 sec). Calibration: 1.0 mV, 5 msec.

**Glutamate facilitates the potentiating effect of Zn²⁺**

Because glutamate is also released from mossy fiber terminals during electrical stimulation, it is reasonable to expect that, under physiological conditions, both glutamate and Zn²⁺ are required...
for LTP induction in these terminals. In our next set of experi-
ments, we sought to verify that simultaneous application of exog-
enous glutamate and Zn$^{2+}$/H11001 together is suf-
ficient to induce long-
lasting potentiation. In these experiments, co-perfusion of 
glutamate and Zn$^{2+}$/H11001, to imitate the co-release of both from 
synaptic terminals, induced long-lasting potentiation (Fig. 6
A) similar to that induced by Zn$^{2+}$/H11001 alone (Fig. 5
A). The mean 
potentiated EPSP slope was 241
$\pm$11\% (mean
$\pm$SEM; n = 6) of 
baseline, and the potentiation was stable for 
3 hr. Glutamate 
alone, however, in the presence of 10 mM CaEDTA induced only 
a transient EPSP potentiation, followed by an immediate return 
to baseline after glutamate washout (Fig. 6A). This result implies 
that glutamate itself could not induce a persistent EPSP potentia-
tion. As summarized in Figure 6B, the effect of Zn$^{2+}$/H11001 on induction 
of long-lasting potentiation was modulated by the level of gluta-
mate in the bath. With the standard low-frequency (0.03 Hz) test 
stimulation, adding both glutamate and Zn$^{2+}$/H11001 caused a more

Figure 4. Exposure of slices to a high concentration of CaEDTA does 
not alter normal basal synaptic responses at mossy fiber→CA3 synapses 
with low-frequency stimulation. A. Free Ca$^{2+}$ was measured in normal 
ACSF (left bar) and in ACSF containing 10 mM CaEDTA (middle bar). 
The right bar shows the concentration of Ca$^{2+}$ measured in ACSF 
containing 10 mM CaEDTA with an additional 0.22 mM CaCl$_2$ (2.4 mM 
CaCl$_2$ in normal ACSF) added to restore free Ca$^{2+}$ to the level in normal 
ACSF. B. Plot of the EPSP against time, with bath-applied CaEDTA (10 
mM) indicated by a dashed line. Each point represents the averaged initial 
spike of evoked EPSP (n = 6). The averaged values were then normalized 
to the mean initial spike during 30 min baseline recording (percent ± 
SEM). The traces on top represent recording before (1) and during (2) 
CaEDTA perfusion. C. The paired pulse ratio is unaffected by the 
addition of CaEDTA, as shown on the left. The bar graph demonstrates 
the average paired pulse ratios from six recordings in which two stimuli 
were given 40 msec apart in the presence and absence of CaEDTA. 
Calibration in B, C: 0.5 mV, 5 msec.

Figure 5. Exogenous Zn$^{2+}$/H11001 induces long-lasting potentiation of the 
EPSP. A. Plot of exogenous Zn$^{2+}$/H11001-induced long-lasting potentiation. 
Zn$^{2+}$/H11001 (100 μM) was applied for 20 min (line) after recording a 30 min 
baseline. Application of Zn$^{2+}$/H11001 was followed by wash with 10 mM 
CaEDTA. Inset, EPSP recorded before and after exposure to Zn$^{2+}$/H11001. Note 
that the afferent volley (arrow) was not affected. B. Percentage of LTP 
duced by different concentrations of exogenous Zn$^{2+}$/H11001. Calculation in A: 
1.0 mV, 5 msec.
rapid EPSP potentiation than did Zn\(^{2+}\) alone. These results indicate that glutamate enables the potentiating effect of Zn\(^{2+}\).

Potentiation of the EPSP requires the entry of Zn\(^{2+}\) into neurons

One plausible explanation for this interaction is that glutamate is required to permit Zn\(^{2+}\) entry into pyramidal neurons. Glutamate allows Zn\(^{2+}\) to enter both directly through Ca\(^{2+}\)-permeable AMPA/kainate and NMDA receptors and indirectly, via its depolarizing effects, through VDCC (Choi and Koh, 1998; Weiss and Sensi, 2000). We directly tested whether glutamate mediates Zn\(^{2+}\) translocation across the postsynaptic membrane by the following experiments. Cells in the slice were loaded with Newport Green diacetate (the cell-permeable dye that, once inside the cell, is hydrolyzed by cytoplasmic esterases to become membrane-impermeable) and then thoroughly washed to eliminate unincorporated dye. Mossy fiber axons were then stimulated with HFS, and the entry of Zn\(^{2+}\) into cells in the CA3 pyramidal layer was imaged. In agreement with our previous findings, HFS caused an immediate influx of Zn\(^{2+}\) into cells (Fig. 7A). The

---

**Figure 6.** Glutamate facilitates the potentiating effect of Zn\(^{2+}\). A, Coincubation of glutamate (100 \(\mu\)M) and Zn\(^{2+}\) (100 \(\mu\)M) expeditiously potentiated EPSPs; potentiation lasted several hours (filled diamonds). Glutamate alone (open squares; in the presence of CaEDTA) failed to induce long-lasting potentiation of the EPSP. Exogenous glutamate and Zn\(^{2+}\) were applied for 20 min (line) after recording a 30 min baseline. Application of glutamate or Zn\(^{2+}\) with glutamate was followed by wash with 10 mM CaEDTA. B, Comparison of the rate of onset of long-lasting potentiation induced by Zn\(^{2+}\) alone and Zn\(^{2+}\) plus glutamate. Each point represents the averaged, normalized EPSP initial slope ± SEM from data in Figure 5A and A, respectively.

**Figure 7.** Increase of intracellular Zn\(^{2+}\) after HFS. A, Paired images of the pyramidal layer of CA3 taken with a 63× water immersion objective lens. Electrical stimulation increased intracellular Zn\(^{2+}\) in the CA3 region of hippocampal slices loaded with Newport Green diacetate (cell-permeable). Before stimulation (I), no cell bodies were labeled; they became visible (2) after stimulation (100 Hz, 500 \(\mu\)A for 10 sec). Scale bar, 50 \(\mu\)m; arrowheads indicate pyramidal cell bodies. B, Intracellular Zn\(^{2+}\) was detected with the fluorescence indicator Newport Green diacetate in normal ACSF and ACSF containing CaEDTA (10 mM) or CNQX (10 \(\mu\)M). The results are plotted on the left. the arrow indicates the beginning of stimulation. The bar graph on the right summarizes the effects of CaEDTA (n = 5) and CNQX (n = 3) on the increase in intracellular Zn\(^{2+}\) after electrical stimulation, expressed as percentage of control. Error bars indicate SEM.
increase of intracellular Zn\textsuperscript{2+} fluorescence elicited by HFS was depressed by addition of either CaEDTA (10 mm) or CNQX (10 \(\mu M\)), an antagonist of AMPA/kainate receptors (Fig. 7B). The remaining fluorescence in the presence of 10 mm CaEDTA was likely caused by rapid entry of a small fraction of released Zn\textsuperscript{2+} into postsynaptic neurons or into presynaptic terminals. CNQX can block entry of Zn\textsuperscript{2+} not only through AMPA/kainate receptors but also through VDCC and NMDA receptors by preventing membrane depolarization by AMPA/kainate receptor channels. These results agree with our finding that CaEDTA blocks HFS-induced LTP by blocking Zn\textsuperscript{2+} entry into postsynaptic neurons.

Because CNQX blocked both Zn\textsuperscript{2+} translocation and LTP induction, we tested whether the introduction of Zn\textsuperscript{2+} into neurons by a different route could restore LTP. In several experiments, Zn\textsuperscript{2+} entry via ionotropic glutamate receptors and VDCC was blocked by 10 \(\mu M\) CNQX and 50 \(\mu M\) AP-5 (the NMDA receptor antagonist AP-5 was present in the perfusate throughout these experiments) but allowed to enter via sodium pyritidine, a selective Zn\textsuperscript{2+} ionophore (Zalewski et al., 1993), which directly increases intracellular Zn\textsuperscript{2+} (Fig. 8A). After washout of CNQX, Zn\textsuperscript{2+}, and Na-pyritidine, a long-lasting potentiation developed in the absence of HFS (158 \(\pm\) 12\%, mean \(\pm\) SEM; \(n = 3\)). In contrast, without Na-pyritidine, addition of Zn\textsuperscript{2+} alone in the presence of CNQX failed to induce long-lasting potentiation of the EPSP (93 \(\pm\) 9\%, mean \(\pm\) SEM; \(n = 4\); Fig. 8B). Likewise, pyritidine (50 \(\mu M\)) alone, in the absence of Zn\textsuperscript{2+}, had no long-lasting effect on the EPSP (104 \(\pm\) 5\%, mean \(\pm\) SEM; \(n = 5\); data not shown). In slices loaded with intracellular Newport Green, which could also function as a selective intracellular Zn\textsuperscript{2+} chelator, the induction of LTP by HFS was blocked (110 \(\pm\) 4.5\%, mean \(\pm\) SEM; Fig. 9). These data indicate that the translocation of extracellular Zn\textsuperscript{2+} into postsynaptic neurons is critical for the induction of LTP in mossy fiber→CA3 synapses and suggest an intracellular site of action for Zn\textsuperscript{2+}.

**DISCUSSION**

Our results establish that synaptically released Zn\textsuperscript{2+} plays an essential role in the induction of LTP in mossy fiber→CA3 synapses. Effective Zn\textsuperscript{2+} chelation blocked the induction of LTP by HFS. Bath application of exogenous Zn\textsuperscript{2+} induced a long-lasting potentiation of the EPSP, apparently by acting at an intracellular site rather than at an extracellular site. Co-released glutamate is also essential for induction of LTP under physiological conditions, in part because it allows Zn\textsuperscript{2+} entry into postsynaptic neurons.

The mossy fiber→CA3 synapse contains high concentrations of Zn\textsuperscript{2+} in large synaptic boutons (Haug, 1967; Perez-Clausell and Danscher, 1985; Fredericksion, 1989). This Zn\textsuperscript{2+} is released in the same manner as a neurotransmitter and is thought to be co-released with glutamate on stimulation of presynaptic terminals. We found that Zn\textsuperscript{2+} release is dependent on stimulation frequency (Li et al., 2001). Each mossy fiber bouton terminates on the proximal portion of apical CA3 dendrites with up to 35 release sites (Chicurel and Harris, 1992). Mossy fiber synapses show tremendous frequency facilitation that leads to plateau depolarizations during long stimulus trains (Vogt et al., 2000). Although we do not know the exact relationship between the prominent presence of Zn\textsuperscript{2+} and these unusual features, it indicates that these synapses are capable of rapidly releasing large amounts of Zn\textsuperscript{2+} during HFS. Therefore, it is not surprising that a high concentration of Zn\textsuperscript{2+} chelator was needed to buffer effectively the Zn\textsuperscript{2+} released during the HFS.

Figure 8. Potentiation of the EPSP requires entry of Zn\textsuperscript{2+} into cells. A, Paired images of the pyramidal layer of CA3 (taken with a 63x objective lens) loaded with Newport Green diacetate before (1) and after (2) exposure to Zn\textsuperscript{2+} (100 \(\mu M\)), Na-pyritidine (50 \(\mu M\)), and CNQX (10 \(\mu M\)). Scale bar, 50 \(\mu M\); arrowheads indicate pyramidal cell bodies. B, Plot of the EPSP slope from a representative experiment to show that selectively increasing intracellular Zn\textsuperscript{2+} potentiates the EPSP (158 \(\pm\) 12\%; \(n = 4\); filled diamonds). Zn\textsuperscript{2+} (100 \(\mu M\)) and Na-pyritidine (50 \(\mu M\)) were applied after the EPSP was blocked by CNQX (10 \(\mu M\)). In a similar experiment without Na-pyritidine, no EPSP potentiation was observed (93 \(\pm\) 9\%; \(n = 4\); open squares). Lines indicate the duration of drug application. Inset, Summary of four experiments in which slices were treated with Na-pyritidine and Zn\textsuperscript{2+}. Values of the EPSP amplitude were taken from baseline (1), in the presence of CNQX (2), and during EPSP potentiation (3). Numbers correspond to those in B. *Significant difference (\(p < 0.05\)) between 1 and 3.

In this investigation of the possible role of Zn\textsuperscript{2+} in mossy fiber→CA3 synaptic plasticity, the removal of synaptically released Zn\textsuperscript{2+} with 10 mm CaEDTA blocked the induction of LTP. Direct fluorescence imaging showed that 10 mm CaEDTA chelated 85\% of the synthetically released Zn\textsuperscript{2+}. This result verifies that the effect of CaEDTA on LTP induction in mossy fiber→CA3 synapses was achieved by its selective chelation of the synthetically released Zn\textsuperscript{2+} from mossy fiber terminals. On the other hand, a lower concentration of CaEDTA (1 mm) failed to reduce the extracellular Zn\textsuperscript{2+} after HFS, as evidenced by its inability to reduce Newport Green fluorescence. We have calculated that, under equilibrium conditions, 1 and 10 mm CaEDTA should indeed reduce 10–100 \(\mu M\) Zn\textsuperscript{2+} to 0.04–4 \(nM\). The presence of 2.4 mm Ca\textsuperscript{2+} and 1.3 mm Mg\textsuperscript{2+} in the medium and...
the slow dissociation rate of CaEDTA, however, limit the rate at which Zn$^{2+}$ is chelated (see Materials and Methods and Appendix). Kinetic calculations indicate that Zn$^{2+}$ is chelated in a biphasic manner. The initial rate of chelation, limited by the initial concentration of free EDTA and rapidly dissociating MgEDTA, reaches completion in $<$0.1 msec. The slower phase, limited by the dissociation of CaEDTA, requires many seconds to reach completion at the equilibrium concentrations. Thus 1 mM CaEDTA was unable to prevent the micromolar accumulations of Zn$^{2+}$ induced by HFS from mossy fiber terminals and entry into CA3 dendrites that we have observed to take place within milliseconds, because it provided a lower concentration of free EDTA and MgEDTA relative to the concentration of released Zn$^{2+}$. Ten micromolar CaEDTA, on the other hand, provided enough free EDTA and MgEDTA to reduce the 10–100 μM Zn$^{2+}$ released by the mossy fiber terminals to concentrations below the level readily detected by Newport Green fluorescence as well as those required for LTP.

In the present study, the addition of 50–100 μM exogenous Zn$^{2+}$ to the solution bathing the slice was required to induce a reliable, long-lasting potentiation of the EPSP. These data suggest that Zn$^{2+}$ is able to enhance synaptic strength at mossy fiber–CA3 synapses. Ten micromolar Zn$^{2+}$ did not appreciably alter synaptic transmission. Hence, it can be assumed that under stimulation conditions necessary to induce LTP, concentrations of $>$10 μM Zn$^{2+}$ are released in the synapse. In addition, this may explain why the removal of Zn$^{2+}$ released into the synaptic cleft during basal conditions or low-frequency stimulation failed to alter synaptic transmission. However, Zn$^{2+}$ is able to inhibit the NMDA receptor at concentrations as low as 50 nm (Chen et al., 1997) and may play an important role in shaping the NMDA receptor response at this synapse under normal physiological conditions. Our results confirm the previous observation that, although a deficiency of bouton Zn$^{2+}$ in rats resulted in the impairment of mossy fiber LTP, it does not appear to affect normal synaptic transmission (Lu et al., 2000). Thus, synaptically released Zn$^{2+}$ appears to have little effect on basal synaptic function other than in modulating NMDA responses (Peters et al., 1987; Westbrook and Mayer, 1987; Chen et al., 1997) but is required for LTP induction by HFS in mossy fiber–CA3 synapses, which does not require NMDA receptor activation (Harris and Cotman, 1986). It is possible that Zn$^{2+}$ may act on GABAergic interneurons resulting in an indirect effect on the pyramidal cell. Zn$^{2+}$ inhibits hippocampal postsynaptic GABA current with a $K_{d}$ of 11 μM (Mayer and Vytklicky, 1989). In our study, however, the removal of released Zn$^{2+}$ or the addition of 10 μM exogenous Zn$^{2+}$ did not alter synaptic transmission in mossy fiber–CA3 synapses. Furthermore, Zn$^{2+}$ did not affect the afferent volley in our experimental conditions. We have therefore concluded that the indirect effects of Zn$^{2+}$ acting on interneurons did not inhibit LTP at this synapse.

Because glutamate is also released from mossy fiber terminals during electrical stimulation, it is reasonable to expect that, under physiological conditions, both glutamate and Zn$^{2+}$ are required for LTP induction in these terminals. The results from the present study indicate that glutamate promotes Zn$^{2+}$ entry into the neuron by opening Zn$^{2+}$-permeable channels. It is known that glutamate allows Zn$^{2+}$ to enter cultured neurons directly through Ca$^{2+}$-permeable AMPA/kainate and NMDA receptors and indirectly, via its depolarizing effects, through VDCC and NMDA receptors by preventing membrane depolarization through the activation of AMPA/kainate receptors. We can rule out NMDA receptors out, because we included APV in the ACSF. Toth et al. (2000) have reported that Ca$^{2+}$-permeable AMPA receptors are not expressed in CA3 principal neurons, raising doubt that these channels contribute significantly to Zn$^{2+}$ entry in this region. Kainate receptors or VDCC remain as primary candidates. The possibility that these are the routes of Zn$^{2+}$ entry in the proximal dendrites of CA3 pyramidal neurons in our experiments is also supported by the presence of a high density of VDCCs and putative Ca$^{2+}$-permeable kainate receptors in mossy fiber–CA3 synapses (Westenbroek et al., 1990; Bortolotto et al., 1999; Sui and Ruan, 2000). Ca$^{2+}$ can enter the CA3 pyramidal neurons through these same channels. An increase in intracellular Ca$^{2+}$ in postsynaptic neurons during LTP induction has been established for all hippocampal synapses except the mossy fiber–CA3 synapse (Zalutsky and Nicoll, 1990). Whether Ca$^{2+}$ is required in the induction of mossy fiber LTP is the subject of many debates. Other groups have provided evidence that there is an initial rise in postsynaptic intracellular Ca$^{2+}$ during LTP induction at the mossy fiber synapse (Yeckel et al., 1999). In these reports, LTP was prevented by chelation of postsynaptic intracellular Ca$^{2+}$. Interpretation of these results, however, is complicated by the fact that both the Ca$^{2+}$ indicator and chelator used in those studies have higher affinities for Zn$^{2+}$ than for Ca$^{2+}$. It is impossible to exclude in these studies the role of Zn$^{2+}$ in LTP induction. A delineation of the separate roles of Zn$^{2+}$ and Ca$^{2+}$ in LTP induction and the possible interactions between these two ions will require further investigation.

One of the hallmarks of the mossy fiber synapse is its apparent lack of NMDA receptor-dependent synaptic plasticity. Some re-
ports have indicated that LTP at mossy fiber→CA3 synapses may be of presynaptic origin (Harris and Cotman, 1986; Nicoll and Malenka, 1999). However, in other studies, mossy fiber LTP has required both presynaptic and postsynaptic activation (Jaffe and Johnston, 1990). Many of these data could be explained by such factors as the type of LTP-inducing stimulus applied and the recording conditions, but the reasons for the conflicting results are still unclear. Although examining the presynaptic versus postsynaptic origin of LTP was not the goal of the present study, our results would support either a presynaptic or a postsynaptic origin of LTP. On release, Zn²⁺ could be taken up by selective high-affinity Zn²⁺ transporters in the mossy fiber terminals or could enter the postsynaptic neuron through glutamate and VDCC for induction of mossy fiber LTP. Our results cannot eliminate either mechanism for several reasons: First, CaEDTA prevented Zn²⁺ from entering both presynaptic terminals and postsynaptic neurons. Second, bath-applied Zn²⁺ could enter both mossy fiber terminals and CA3 neurons. Third, although CNQX blocked postsynaptic entry of Zn²⁺, there may still be a substantial amount of Zn²⁺ taken up by presynaptic terminals, as indicated by the difference in the intracellular Newport Green fluorescence obtained in the presence of CaEDTA compared with that obtained with CNQX blockade after HFS. These arguments do not negate our observation, however, that Zn²⁺ must enter cells to perform its role in LTP induction through interactions with kinases, phosphatases, and other intracellular signaling pathways.

Our results indicate the idea that Zn²⁺ released from mossy fiber synapses acts as a presynaptically released second messenger or trans-synaptic factor. A presynaptically released factor that enhances synaptic strength could improve specificity and efficacy of synaptic transmission. In addition to its crucial role for gene expression and transcription, Zn²⁺ has been shown to activate a number of protein kinases such as protein kinase C (Hubbard et al., 1991; Quest et al., 1992), Ca/calmodulin kinase II (Brewer et al., 1979; Weinberger and Rostas, 1991; Lengyel et al., 2000), and mitogen-activated protein kinase (Park and Koh, 1999), which are associated with establishing LTP (Feng, 1995; Soderling and Derkach, 2000; Sweatt, 2001). Mossy fiber boutons terminate on the proximal portion of apical dendrites of CA3 pyramidal neurons. This unusual structure may give Zn²⁺ direct access to modulate gene transcription. Additionally, nanomolar Zn²⁺ signals modulate protein-tyrosine phosphatases and, thus, the phosphorylation of myriad postsynaptic proteins (Maret et al., 1999). Therefore, the present study raises the intriguing possibility that entry of synaptically released Zn²⁺ modulates intracellular signaling pathways and gene transcription.

**APPENDIX**

**Calculations of Zn²⁺ chelation by CaEDTA in the presence of the ions in ACSF.**

Our modeling of Zn²⁺ chelation by CaEDTA in the presence of the Ca²⁺ and Mg²⁺ in ACSF included both equilibrium and kinetics calculations. Although 1 mM CaEDTA may be adequate to remove released free Zn²⁺ by thermodynamic arguments, the kinetics of chelation may be too slow to achieve adequate removal within the time frame of a synaptic event. Equilibrium constants were obtained from Martell and Smith (1974) and Bers et al. (1994). Kinetic constants were obtained from Davis et al. (1999) and Hering and Morel (1988) (Table 1). In the absence of an experimentally derived on-rate for Zn²⁺ complexing with EDTA, we used the rate of diffusion (10³ m/sec) as the rate that limits Zn²⁺–EDTA complex formation. The off-rate constant was then calculated from the Kᵣ. The equations used for the equilibrium case are as follows:

\[ CE = C_T - C, \quad ME = M_T - M, \quad ZE = Z_T - Z. \quad (1) \]

Total EDTA in solution is given by:

\[ E_T = E + CE + ME + ZE. \quad (2) \]

Substituting the chelated forms for the expressions containing total concentrations in Equation 1 and rearranging, the total concentrations on the left:

\[ E_T - C_T - M_T - Z_T = E - C - M - Z. \quad (3) \]

\[ K_T = E_T - C_T - M_T - Z_T. \quad \text{Then,} \quad K_T = E - C - M - Z. \quad (4) \]

The proximal portion of apical dendrites of CA3 pyramidal neurons. This unusual structure may give Zn²⁺ direct access to modulate gene transcription. Additionally, nanomolar Zn²⁺ signals modulate protein-tyrosine phosphatases and, thus, the phosphorylation of myriad postsynaptic proteins (Maret et al., 1999). Therefore, the present study raises the intriguing possibility that entry of synaptically released Zn²⁺ modulates intracellular signaling pathways and gene transcription.

**Table 1. Kinetic constants used in calculating the Zn²⁺ concentration values in Figure 3**

<table>
<thead>
<tr>
<th>Ion</th>
<th>( k_{\text{on}} ) (m/sec)</th>
<th>( k_{\text{off}} ) (m/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺</td>
<td>( 8.75 \times 10^5 )</td>
<td>2.8</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>( 2.20 \times 10^7 )</td>
<td>0.7</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>( 1 \times 10^6 )</td>
<td>( 7 \times 10^{-6} )</td>
</tr>
</tbody>
</table>

**Table 2. Equilibrium constants and Zn²⁺ concentration in ACSF at two different concentrations of total Zn²⁺ and CaEDTA**

<table>
<thead>
<tr>
<th>[Zn²⁺] ( (\mu M) )</th>
<th>[CaEDTA] ( (\mu M) )</th>
<th>( K_{\text{Zn²⁺}} )</th>
<th>( K_{\text{Ca}^{2+}} )</th>
<th>( K_{\text{Mg}^{2+}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>10</td>
<td>( 3.68 \times 10^{-11} )</td>
<td>( 4.16 \times 10^{-14} )</td>
<td>( 3.01 \times 10^{-8} )</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>( 3.55 \times 10^{-9} )</td>
<td>( 4.00 \times 10^{-14} )</td>
<td>( 2.9 \times 10^{-8} )</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>( 3.53 \times 10^{-11} )</td>
<td>( 4.16 \times 10^{-14} )</td>
<td>( 3.01 \times 10^{-8} )</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>( 3.21 \times 10^{-10} )</td>
<td>( 4.00 \times 10^{-14} )</td>
<td>( 2.9 \times 10^{-8} )</td>
</tr>
</tbody>
</table>

At 32°C, pH 7.4, ionic strength 0.159.
Rearranging the definition of the dissociation constants and substituting Equation 1 for the chelated forms:

\[ E = K_C \cdot (C_T - C)/C, \quad E = K_M \cdot (M_T - M)/M, \quad E = K_Z \cdot (Z_T - Z)/Z, \]

we can derive three equations by substituting Equation 5 in Equation 4:

\[ K_T = (K_C \cdot (C_T - C)/C) - C - M - Z, \]

\[ K_T = (K_M \cdot (M_T - M)/M) - C - M - Z, \]

\[ K_T = (K_Z \cdot (Z_T - Z)/Z) - C - M - Z. \]

Equations 6–8 are quadratic equations, each with three unknowns. They could be solved simultaneously but we used an iterative calculation method using Microsoft Excel. Estimates were made of each ion concentration initially in the absence of Zn\(^{2+}\) and then including Zn\(^{2+}\), and a calculated value was determined using Equations 6–8, in order of increasing affinity for EDTA. With each calculation, the calculated value was substituted for the estimated made for that ion. A macro was created to do this iteratively until the difference between the calculated and estimated values for Mg\(^{2+}\) concentration was less than an arbitrary critical value \((1 \times 10^{-7})\). The results for Zn\(^{2+}\) concentration are shown in Table 2. The equilibrium values for Ca\(^{2+}\), Mg\(^{2+}\), and Zn\(^{2+}\) agreed with those calculated by WEBMAXC version 2.10 (www.stanford.edu/~cpatton/webmax2.htm) using the same equilibrium constants (Table 2). Our estimates of equilibrium constants assumed a temperature of 32°C, pH 7.40, and an ionic strength of 0.159 for ACSF.

The kinetics of Zn\(^{2+}\) chelation were calculated as follows: Let \( k_{\text{off}} \) and \( k_{\text{on}} \) be the dissociation and association rate constants. Then:

\[ \frac{dZ}{dt} = k_{\text{on}} \cdot Z \cdot E - k_{\text{off}} \cdot ZE = k_{\text{on}} \cdot Z \cdot E - k_{\text{off}} \cdot (Z_T - Z). \]

Substituting Equation 4 for \( E \) in Equation 9, the rate of zinc chelation can be calculated from the concentrations of the three metal ions and other constants:

\[ \frac{dZ}{dt} = k_{\text{on}} \cdot Z \cdot (K_T + C + M + Z) - k_{\text{off}} \cdot (Z_T - Z). \]

This equation is also quadratic. The equations for the other ions were derived in the same way and have the same form. Again, we used an Excel spreadsheet to calculate an approximation of the concentrations of each of the three metal ions over time from initial conditions using an appropriately small interval \((1 \times 10^{-7}\) sec). The initial conditions chosen were consistent with our experimental conditions. These take the concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) in ACSF to be those at equilibrium with the given concentration of CaEDTA in the absence of Zn\(^{2+}\). The given concentration of Zn\(^{2+}\) was assumed to be released instantaneously into the medium at time 0. Figure 3 shows the time course of Zn\(^{2+}\) concentration change over the course of 0.1 msec, a time frame considered typical for neurotransmitter release.

REFERENCES


Palmiter RD, Cole TB, Quaife CJ, Findley SD (1996) ZnT-3, a putative mossy fiber-CA3 LTP Requires Translocation of Released Zn\(^{2+}\).


Zalewski PD, Forbes IJ, Betts WH (1993) Correlation of apoptosis with change in intracellular soluble \( Zn^{2+} \) using zinquin ([2-methyl-8-p-toluenesulphonamido-6-quinolyl)oxycetic acid], a new specific fluorescent probe for \( Zn^{2+} \). Biochem J 296:403–408.