

RESEARCH ARTICLE

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Zinc release from thapsigargin/IP₃-sensitive stores in cultured cortical neurons

Christian J Stork¹ and Yang V Li^{*1,2,3}

Abstract

Background: Changes in ionic concentration have a fundamental effect on numerous physiological processes. For example, IP₃-gated thapsigargin sensitive intracellular calcium (Ca²⁺) storage provides a source of the ion for many cellular signaling events. Less is known about the dynamics of other intracellular ions. The present study investigated the intracellular source of zinc (Zn²⁺) that has been reported to play a role in cell signaling.

Results: In primary cultured cortical cells (neurons) labeled with intracellular fluorescent Zn²⁺ indicators, we showed that intracellular regions of Zn²⁺ staining co-localized with the endoplasmic reticulum (ER). The latter was identified with ER-tracker Red, a marker for ER. The colocalization was abolished upon exposure to the Zn²⁺ chelator TPEN, indicating that the local Zn²⁺ fluorescence represented free Zn²⁺ localized to the ER in the basal condition. Blockade of the ER Ca²⁺ pump by thapsigargin produced a steady increase of intracellular Zn²⁺. Furthermore, we determined that the thapsigargin-induced Zn²⁺ increase was not dependent on extracellular Ca²⁺ or extracellular Zn²⁺, suggesting that it was of intracellular origin. The applications of caged IP₃ or IP₃-3Kinase inhibitor (to increase available IP₃) produced a significant increase in intracellular Zn²⁺.

Conclusions: Taken together, these results suggest that Zn²⁺ is sequestered into thapsigargin/IP₃-sensitive stores and is released upon agonist stimulation.

Background

Zn²⁺ is an important structural and functional component in many cellular proteins and enzymes. As such, Zn²⁺ levels are normally tightly regulated, limiting the extent of cytosolic labile (or free) Zn²⁺ concentrations [1,2]. For example, levels of free Zn²⁺ are several orders of magnitude less than that of Ca²⁺ [3]. Zn²⁺ may act as a cellular messenger in physiological and cytotoxic signaling, and the changes in Zn²⁺ homeostasis have a fundamental effect in cell function [4,5]. Many studies have shown the accumulation of excessive Zn²⁺ to precede cell death or neurodegeneration in response to cytotoxic stress [6,7]. To characterize Zn²⁺-mediated signaling pathways or Zn²⁺-induced cytotoxicity, it is important to determine the source(s) of intracellular free Zn²⁺ in response to specific stimuli or injury.

The endoplasmic reticulum (ER) is an intracellular organelle that has been shown to sequester Ca²⁺ from the cytosol by means of sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA) or so-called endoplasmic Ca²⁺ pump [8]. This sequestered Ca²⁺ can be released into the cytosol upon a variety of stimuli including inositol 1,4,5-trisphosphate (IP₃). It is IP₃ that mobilized Ca²⁺ from the ER Ca²⁺ store following interaction with specific IP₃ receptors (IP₃R). A commonly used tool in studying Ca²⁺ homeostasis is thapsigargin, a plant derived compound that specifically inhibits SERCA activity [9]. By blocking the ability of the cell to pump Ca²⁺ into the ER, thapsigargin causes these stores to become depleted and thereby raise the cytosolic Ca²⁺ concentration.

While the mechanisms responsible for regulating Zn²⁺ homeostasis are not well established, available data support that, like Ca²⁺, intracellular Zn²⁺ levels are determined by the interaction of membrane Zn²⁺ transporters and cytoplasmic Zn²⁺ buffers [4,10]. The present study investigates the intracellular source of free Zn²⁺, particu-

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larly, if thapsigargin can trigger the release of Zn^{2+} . This possibility is supported by recent evidence that Zn^{2+} can be released from intracellular sources upon stimulation [11-13]. Our results show that Zn^{2+} is released from thapsigargin-sensitive and IP_3R -mediated stores.

Methods

Primary Cell Culture

Pregnant Sprague-Dawley rats (E17-E18) were anaesthetized with CO_2 and the fetuses were removed and placed in ice-cold Hank's Balanced Salt Solution without Ca^{2+} or Mg^{2+} (HBSS). The brains of fetuses were removed and placed into cold HBSS for further dissection. Using a dissecting microscope and blunt dissection, the meninges were gently separated away. The cerebral cortex was then removed and each cortical hemisphere was cut into four pieces and trypsinized in HBSS at $37^\circ C$. Following trypsinization, cells were separated by trituration through the opening of a fire polished Pasteur pipette. The suspensions were then passed through a $70\ \mu m$ cell strainer. The dissociated cells were added to the bottom of 35mm glass-bottomed petri dishes previously coated with polyethyleneimine (50% solution, Sigma, St. Louis) diluted 1:1000 in borate buffer. The cortical neurons were then allowed to attach to the surface at $37^\circ C$, 5% CO_2 in 2 ml of MEM solution (Gibco, BRL) supplemented with 10% (v/v) heat-inactivated fetal bovine serum. After 3-6 hrs, solutions were replaced with fresh supplemented MEM which was later replaced (24 hrs) with Neurobasal medium (Gibco, BRL) supplemented with 2% B-27.

Fluorescence Microscopy

All imaging experiments were performed in HEPES medium containing the following (in mM): 130 NaCl, 5 KCl, 8 $MgSO_4$, 1 Na_2HPO_4 , 25 Glucose, 20 HEPES, 1 Na-Pyruvate; pH adjusted to 7.4. Cortical cells grown in glass-bottomed petri dishes were washed with fresh HEPES medium. The cells were then incubated at $37^\circ C$ for 30 min with ER-Tracker Red (Molecular Probes, Carlsbad, CA) and Newport Green (Molecular Probes, Carlsbad, CA) or ZinPyr-1 (Neurobiotex, Galveston, TX). Cells were incubated with $10\ \mu M$ of the specified fluorescent Zn^{2+} indicator either alone or in conjunction with ER-Tracker red for 30 min then were washed 3x with fresh HEPES medium and placed into a custom 35mm stage adapter and continuously perfused with fresh HEPES medium on the stage of a Zeiss LSM 510 (confocal) microscope. Cultures were examined using a Plan-Neofluar 100x/1.3 NA oil immersion objective. For ER-Tracker Red excitation was done with a HeNe Laser line of 543nm and an LP560nm emission filter. Newport Green and ZinPyr-1 were imaged using an Argon Laser line of 488nm for excitation and a BP505-550nm emis-

sion filter. Separate fluorescent channels were employed for each indicator, and channels were scanned sequentially to minimize crosstalk. Cells were imaged by serial z-scans progressively from bottom to top, in increments of 500 nm. Colocalization of ER-Tracker Red and Newport Green or ZinPyr-1 was measured using Zeiss software [14]. Colocalization was determined by Pearson's correlation coefficient and considered significant when ($p \leq 0.05$). Time series measurements of fluorescence intensity were done with image capture at 10 sec intervals, and changes in intensity were measured using Zeiss LSM 510 image analysis software. Fluorescence measurements were background subtracted, normalized to starting values, and expressed as F/Fo.

Caged IP_3 experiment

To directly activate IP_3R s we used the membrane-permeable UV light-sensitive caged IP_3 analogue, ci- IP_3/PM (D-2,3-O-isopropylidene-6-O-(2-nitro-4,5-dimethoxy) benzyl-myoinositol 1,4,5-trisphosphate-hexakis(propionoxymethyl)ester (SiChem, Bremen, Germany) [15,16]. Cells in brain hippocampal slices were simultaneously loaded by incubation with caged IP_3 and Newport Green for 30 min at $37^\circ C$, then washed and incubated for an additional 30 min to allow for complete de-esterification.

IP_3 was photoreleased by flashes of 364 nm light focused uniformly throughout the field of view.

Drug Treatments

Thapsigargin (Molecular Probes, Carlsbad, CA), the IP_3R inhibitor N^2 -(m-trifluorobenzyl), N^6 -(p-nitrobenzyl)purine (Calbiochem, Cat. No. 406170), N,N,N',N' -tetrakis (2 pyridylmethyl) ethylenediamine (TPEN) (Molecular Probes, Carlsbad, CA), the $Ins(1,4,5)P_3$ receptor blocker 2-aminoethoxydiphenyl-borate (2-APB), were applied by bath application in HEPES medium.

Results

Intracellular Regions of Elevated Zn^{2+} Co-localize with the Endoplasmic Reticulum

Cells labeled with intracellular fluorescent Zn^{2+} indicators and examined under basal conditions showed consistent regions of elevated fluorescent intensity in the soma and processes, and particularly in a region that was identified as the endoplasmic reticulum by a fluorescent marker for the organelle. The elevated levels of Zn^{2+} were seen to represent labile or free Zn^{2+} because they were sensitive to both low and high affinity fluorescent Zn^{2+} indicators Newport Green ($K_D Zn^{2+} \approx 10^{-6} M$) (Figure 1A) and ZinPyr-1 ($K_D Zn^{2+} \sim 10^{-9} M$) (Figure 1B). Before imaging, cells were washed three times with fresh medium to remove excessive fluorescent residues. Another reason for using ZinPyr-1 is that, unlike AM forms of fluores-

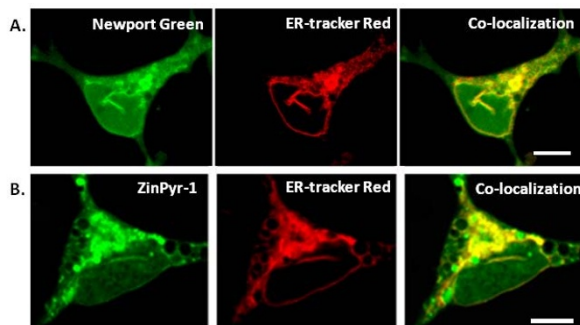


Figure 1 Co-localization of intracellular Zn²⁺ and ER fluorescence. Images of cultured cortical neurons co-labeled with the intracellular fluorescent Zn²⁺ indicators and a live cell marker for the ER. **A.** Fluorescent Zn²⁺ indicator Newport Green AM co-localizes with the ER marker ER-Tracker Red. **B.** Fluorescent Zn²⁺ indicator ZinPyr-1 co-localizes with the ER marker ER-Tracker Red. Cells were incubated with 10 μM of the specified fluorescent Zn²⁺ indicator and 1 μM ER-Tracker Red then imaged using a LSM510 confocal microscope equipped with a 100x/1.3NA objective.

cence indicators, it is highly lipophilic and remains in the organelle, being sequestered with zinc. These indicators are essentially insensitive to Ca²⁺ and their fluorescence to Zn²⁺ are not altered in the presence of Ca²⁺ [17,18]. Fluorescence was sensitive to quenching by the membrane permeable Zn²⁺-chelator TPEN (K_DZn²⁺ ~ 10⁻¹⁷ M) (Figure 2). The same cells were also loaded with ER-tracker Red, a marker for the ER, to determine the localization of the intracellular fluorescent Zn²⁺ indicators. We performed serial z-scans using confocal microscopy of cortical neurons loaded with ER-tracker Red and either Newport Green or ZinPyr-1 (Figure 1A, B). Both fluorescent Zn²⁺ indicators showed significant colocalization

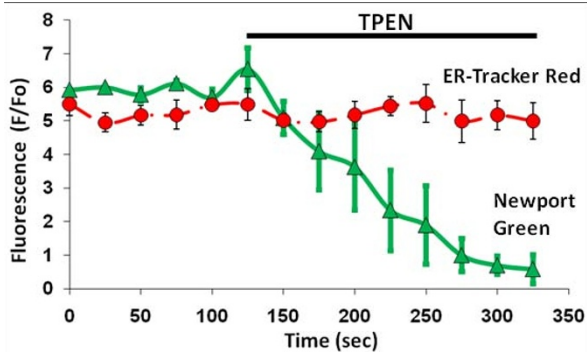


Figure 2 Effect of Zn²⁺ chelation. The graph indicates the fluorescent response of Newport Green (green line) and ER-Tracker Red (red line) in unstimulated neurons upon TPEN (10 μM) exposure, data points represent the mean ± SD of n = 5 trials. The quenching of Newport Green fluorescence with TPEN indicates the presence of free Zn²⁺ in the basal condition.

with the ER Tracker Red. Colocalization was abolished upon exposure to 10 μM TPEN (Figure 2), indicating that the local Zn²⁺ fluorescence represented free Zn²⁺ in the basal condition and were located within the lumen of the ER.

Thapsigargin-Induced Zn²⁺ Release

To assess the overall dynamics of Zn²⁺ release from thapsigargin-sensitive stores, cells incubated with Newport Green were exposed to thapsigargin, which inhibits SERCA pump. The application of thapsigargin depletes the ER Ca²⁺ stores in cells and raises cytosolic Ca²⁺ concentration. The experiments above suggested that Zn²⁺ may be also transported into and sequestered in ER. If this was true, the accumulation of Zn²⁺ by blocking SERCA with thapsigargin should also produce Zn²⁺ signals. Indeed, exposure to thapsigargin resulted in a gradual increase of cytosolic or intracellular Zn²⁺ (Figure 3), which was sensitive to the Zn²⁺ chelator TPEN (Figure 4).

In the next two tests we determined that the source of this thapsigargin-induced Zn²⁺ increases. One possible source is the influx of extracellular Zn²⁺. To remove extracellular Zn²⁺, we applied a membrane impermeable Zn²⁺ chelator CaEDTA. As shown in figure 4, thapsi-

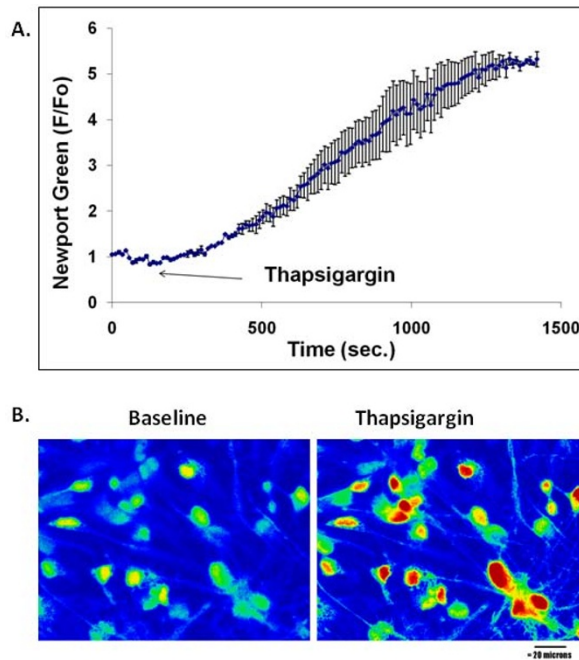
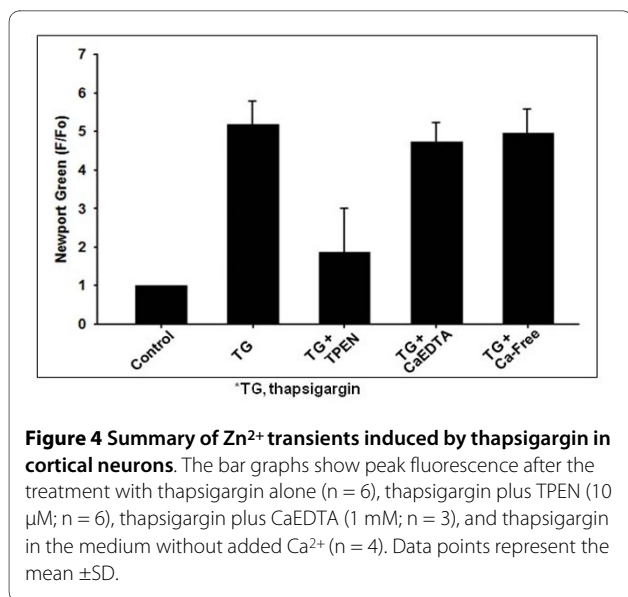


Figure 3 Thapsigargin-induced Zn²⁺ release in cultured cortical neurons. **A.** The graph shows the increases in fluorescence intensity of cells in response to the treatment with thapsigargin (2 μM). Data points represent the mean ± SD of n = 3 trials. Cells were labeled with Newport Green (10 μM). **B.** Representative fluorescent images of cortical neurons loaded with Newport Green before and after exposure to thapsigargin.



gargin-induced Zn²⁺ rises were unchanged in the presence of CaEDTA (1 mM). Next, we examined the contribution of extracellular Ca²⁺ on thapsigargin-induced elevation of intracellular Zn²⁺. We found that the removal of extracellular Ca²⁺ had no effect on the thapsigargin-induced Zn²⁺ rises (Figure 4). Taken together, these results indicated that thapsigargin-induced increases in intracellular Zn²⁺ were not dependent on either extracellular Zn²⁺ or extracellular Ca²⁺, and were entirely of intracellular origin.

IP₃-Induced Intracellular Zn²⁺ Release

The endoplasmic reticulum is a well established site of intracellular Ca²⁺ storage and release. IP₃ can trigger the release of Ca²⁺ from intracellular stores by binding to and activating its receptor (IP₃R) located on regions of the ER. When IP₃ binds to and activates IP₃Rs, the channel portion of the receptor opens and Ca²⁺ is released from the ER to the cytosol. The experiment was therefore performed utilizing ci-IP₃/PM, a cell-permeable form of caged IP₃ to directly induce Zn²⁺ release [15,16,19-21]. In neurons loaded with the Zn²⁺ fluorophore Newport Green and caged IP₃, IP₃ uncaging resulted in a rapid increase in intracellular Zn²⁺, which persisted for 30 s and followed by a roughly exponential decay (Figure 5A & 5B). This experiment demonstrated that the Zn²⁺ response was due to the activation of IP₃ receptors.

A significant route of IP₃ metabolism is the conversion of IP₃ into inositol (1,3,4,5)-tetrakisphosphate (Ins(1,3,4,5)P₄) by the enzyme Ins(1,4,5)P₃ 3-kinase (IP₃-

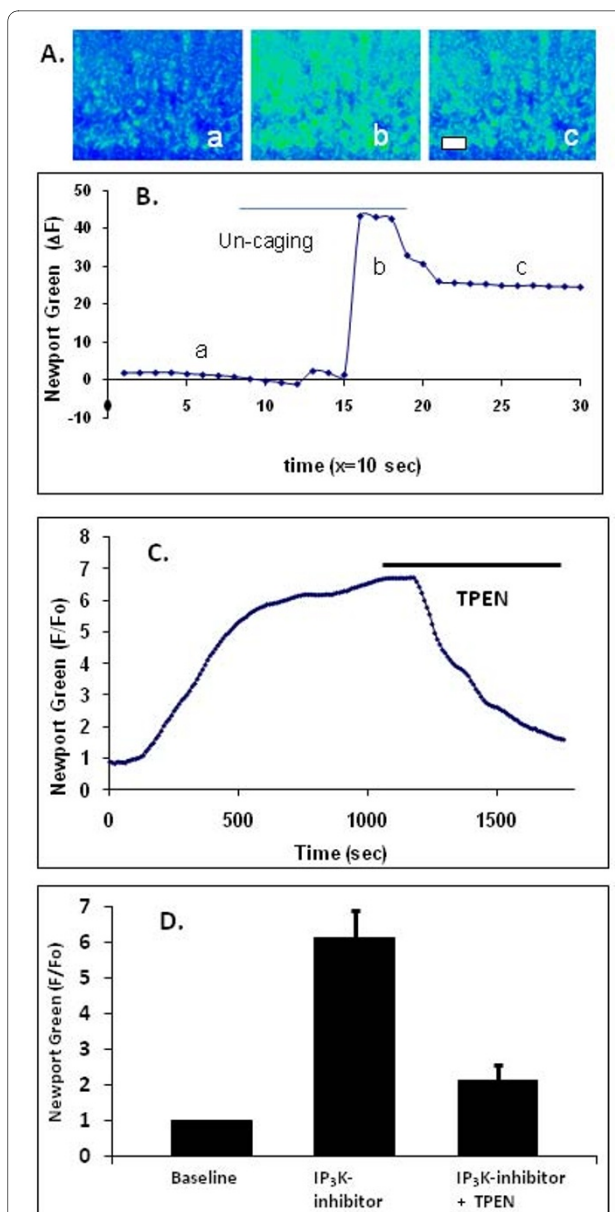


Figure 5 IP₃ stimulation results in the release of intracellular Zn²⁺.

A. The photo-uncaging of caged IP₃ (2 μM) in brain hippocampal neurons loaded with the Zn²⁺ fluorescent indicator Newport Green results in an increase of cytosolic Zn²⁺. Scale bar = 20 μm and the images *a*, *b*, and *c* corresponding to points in B. **B.** The graph shows a change of fluorescence in response to IP₃ stimulation. **C.** A change of fluorescence in responses to the treatments of the IP₃-3K inhibitor N2-(m-trifluorobenzyl),N6-(p-nitrobenzyl)purine (20 μM) and TPEN (10 μM) in cells loaded with Newport Green. **D.** Bar graph shows the average response of Newport Green to the IP₃K-inhibitor (n = 4). The application of TPEN at the peak of fluorescence resulted in significant (n = 2) fluorescence quenching, demonstrating that the fluorescence elevation was due to increased free Zn²⁺.

3Kinase or IP₃-3K [22]. Inhibition of the IP₃-3K has been shown to elevate intracellular levels of IP₃ by halting its conversion into Ins(1,3,4,5)P₄ [23]. Here, to examine the effects of IP₃ signaling on intracellular Zn²⁺ dynamics, N²-(m-trifluorobenzyl),N⁶-(p-nitrobenzyl)purine, a membrane-permeable inhibitor of IP₃-3K was employed to confirm the results observed using the caged IP₃. Upon bath application of the inhibitor, Newport Green fluorescence showed a gradual, significant increase (Figure 5C & 5D), supporting an IP₃ mediated process involved in the release of Zn²⁺.

Discussion

The major findings of the present study are the following: Neuronal cells maintain a substantial concentration of Zn²⁺ in ER-like storage, and Zn²⁺ is released into the cytosol in a thapsigargin- and IP₃-sensitive manner. These findings suggest a new model of intracellular Zn²⁺ homeostasis where cellular organelles like the ER act as sites of intracellular Zn²⁺ storage.

Available data support that intracellular Zn²⁺ levels can be determined by the interaction of membrane Zn²⁺ transporters and cytoplasmic Zn²⁺ buffers [4,10]. In eukaryotic cells the concentration of intracellular Zn²⁺ has been found to be in the range of a few hundred micromolars [24]. The vast majority of this cellular Zn²⁺ is, however, protein bound or sequestered into organelles, which results in free cytosolic Zn²⁺ concentrations being in the picomolar to nanomolar range [10,24,25]. This is consistent with our observations that there is a sharp contrast in Zn²⁺ fluorescence between ER-like lumen and cytosolic space (Figure 1). The significant concentration of free (labile) Zn²⁺ present in ER-like lumen suggests the existence of a high intraluminal Zn²⁺ sequestering activity.

The present study shows that Zn²⁺ is released from thapsigargin-sensitive and IP₃R-mediated stores (Figure 3 & 5). Thapsigargin, as expected, also induced a Ca²⁺ transient measured with a fluorescent Ca²⁺ indicator (data not shown but see [26-28]). Collectively, we suggest that Ca²⁺ is not the only metal ion that is sequestered in the ER. Zn²⁺ is released alongside of Ca²⁺ upon thapsigargin stimulation. We show further that the source of thapsigargin-induced elevation in intracellular Zn²⁺ is of intracellular origin. The elevation of Zn²⁺ is independent from either extracellular Ca²⁺ or extracellular Zn²⁺. These results indicate that Zn²⁺ homeostasis, like Ca²⁺ homeostasis, is controlled by IP₃Rs (Figure 5) that may gate Zn²⁺ into the cytoplasm, and by thapsigargin sensitive ATPase activity that pumps Zn²⁺ from the cytoplasm into the ER.

Within the ER, it is known that Ca²⁺ is buffered by the abundant luminal resident chaperone protein calreticulin

which binds Ca²⁺. Although calreticulin was first identified as a Ca²⁺ binding protein [29], this protein is multi-functional [30] and binds other ions including Zn²⁺ with multiple binding sites [31-34]. Zn²⁺ also binds with several other luminal proteins [35]. Just like Ca²⁺, recent work indicates that mitochondria take up cytosolic Zn²⁺ and that Zn²⁺ accumulation leads to a loss of mitochondrial membrane potential (see review [36]). There are reports suggesting that thapsigargin/IP₃ regulate mitochondrial Ca²⁺ signaling and function [37]. It remains to be studied how thapsigargin and IP₃ induced Zn²⁺ release affect mitochondrial function.

While the mechanism(s) that govern Zn²⁺ trafficking remain elusive, there is little doubt that the intracellular free Zn²⁺ level must be maintained within a physiological limit. Zn²⁺ has been shown to activate a number of protein kinases such as protein kinase C, CaMKII, TrkB, Ras and MAP kinase [38-43]. On the other hand, abnormal levels of Zn²⁺ may lead to either Zn²⁺-induced toxicity or Zn²⁺ deficiency-induced apoptosis [5]. Therefore, thapsigargin sensitive storage or the ER may function as a source of intracellular free Zn²⁺ in response to stimuli, and is likely to play an important role in the regulation of intracellular levels of Zn²⁺.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CS carried out the fluorescence imaging experiments, analysed data, and participated in the experimental design and the preparation of manuscript. YL conceived of the study, and participated in its design, and drafted and prepared manuscript, and coordination. All authors read and approved the final manuscript.

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