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Determining zinc with commonly used calcium and zinc fluorescent indicators, a question on calcium signals

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Abstract

Investigations into the roles of Ca^{2+} and Zn^{2+} in cell biology have been facilitated by the development of sensitive fluorometric probes that have enabled the measurement of Ca^{2+} or Zn^{2+} in both extracellular and intracellular environments. It is critical to be aware of the specificity and relative selectivity of a probe for the targeted ion. Here, we investigated metal-ion responses by screening nominally Zn^{2+}- or Ca^{2+}-selective fluorophores in solutions containing various concentrations of Ca^{2+}, as a potential interferent for Zn^{2+}, or Zn^{2+}, as a potential interferent for Ca^{2+}. The results suggested that Zn^{2+}-sensitive dyes were more specific for their targeted ion than dyes that targeted Ca^{2+}. Ca^{2+}-sensitive dyes such as Calcium Green-1, Fura-2, and Fluo-3 showed a wide range of interaction with Zn^{2+}, even responding to Zn^{2+} in the presence of high concentrations of Ca^{2+}. We demonstrate that these Ca^{2+} indicators can effectively measure dynamic changes of cytosolic Zn^{2+}. Our results appeal for a new generation of Ca^{2+} fluorophores that are more specific for Ca^{2+} over Zn^{2+}. One implication of these results is that data obtained using Ca^{2+}-sensitive dyes may need to be re-examined to determine if results previously attributed to Ca^{2+} could, in part, be due to Zn^{2+}.

Keywords: Zinc; Calcium; Fluorescence; Fluorophore; Calcium Green-1; Fura-2; Fluo-3; Newport Green; FluoZin-3; Zinpyr-4

1. Introduction

Accumulated evidence shows that changes in ion concentration affect numerous processes in brain function. For example, Ca^{2+} plays an important role in regulating numerous neuronal processes, including excitability, neurotransmitter release, gene transcription, cell proliferation, and synaptic plasticity [1,2]. Moreover, excessive Ca^{2+} influx or Ca^{2+} overloading may contribute to neuronal death after ischemic insults or brain trauma [3–6]. Changes in Na^+ and K^+ concentration through widely distributed Na^+ and K^+ channels have also been implicated in regulating neuronal function [7,8].

There is increasing evidence implicating transition metal ions at all levels of cellular function: as catalysts, structural elements, and possibly as second messengers. In particular, the non-redox active transition metal Zn^{2+}, an essential co-factor for many enzymes and other proteins [9], has recently emerged as a candidate cellular messenger in physiological and cytotoxic signaling [10,11]. In the CNS, Zn^{2+} is important for brain development and maintaining an appropriate Zn^{2+} concentration is critical to brain function. Zn^{2+} deficiency leads to mental retardation and long-term behavioral changes in humans [12,13]. Moreover, excessive exposure of neurons to divalent Zn^{2+} can be toxic [14], inducing apoptosis at lower concentrations [15,16] and necrosis at higher concentrations [16–18].

Zn^{2+} exists in biological systems as bound Zn^{2+} and histochemically reactive free Zn^{2+} (chelatable Zn^{2+}). Bound zinc, which makes up the majority of the total zinc in tissues, is comprised largely of metalloenzyme- and metalloprotein-bound zinc [18]. In this form, Zn^{2+} usually serves as an essential catalytic or structural element of zinc-binding proteins. Zn^{2+} may be released from such proteins during oxidative stress [10,19,20]; however, the pathophysiological role of this Zn^{2+} release is not well understood. In animal models, Zn^{2+} accumulates in neurons destined to die after ischemia, seizure, and blunt trauma [21–23]. Although the precise

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source of Zn^{2+} released under these conditions is not well understood, it is clear that these injury paradigms involve Zn^{2+}mobilization and redistribution. Certain neurons contain large amounts of vesicularized Zn^{2+}, which represents up to 10% of the total zinc in the brain and is found mostly in synaptic vesicles of a subgroup of glutamatergic neurons [24]. The specific function of vesicular Zn^{2+} is not yet clear; its release and accumulation in neurons during synaptic activity suggests that it may play a role in neurotransmission [25,26] or neuronal plasticity [27].

Much of our current knowledge of the roles of Ca^{2+} as a second messenger comes from its interaction with fluorescent indicators. The emerging evidence for increases in intracellular Zn^{2+} during neural activities thus poses a dilemma: since almost all fluorescent Ca^{2+} indicators also bind Zn^{2+} with high affinity [28,29], elevation of intracellular Zn^{2+} could comprise part of conventionally measured intracellular Ca^{2+} signals. For example, although best known as an excitation wavelength-ratiometric indicator for Ca^{2+}, Fura-2 is actually a hundred-fold more sensitive as an indicator for Zn^{2+} [28]. While the search continues for high affinity Ca^{2+} fluorescent indicators, the potential problems of their sensitivity to Zn^{2+} and of interference by Zn^{2+} have been largely ignored.

Just as fluorescent Ca^{2+} probes have facilitated research into the dynamics of Ca^{2+} signaling, investigation of the role of Zn^{2+} in cellular processes has been facilitated by the development of sensitive fluorometric probes that have enabled the measurement of Zn^{2+} in both extracellular and intracellular environments. However, measuring Zn^{2+} in cells and environmental samples with fluorescent indicators originally designed for the detection of Ca^{2+} is complicated by competitive binding of other cations, namely Ca^{2+} itself. Thus, in exploring the role of metal ions such as Zn^{2+} and Ca^{2+} it is important to both ensure the specificity and selectivity of the probe to the target ion and to control potential interferent ions in the test solution.

Properties such as the selectivity and sensitivity (range of detection) of a metallofluorescent probe dictate the outcome of ion measurement. These properties are influenced by factors in the experimental environment such as constituents in the test medium. For example, substantial amounts of Ca^{2+}, which is present in both extracellular and intracellular spaces, could interfere with the accuracy of Zn^{2+} measurements. Given the production of new probes in recent years, it is essential to examine the properties of a given probe in a given experimental environment. Here, we have investigated metal ion responses by screening fluorescent Zn^{2+}- or Ca^{2+}-selective indicators in solutions containing various concentrations of potential interferents, such as Ca^{2+} or Zn^{2+} themselves. The objective of this study was to compare the sensitivity and selectivity of several commonly used fluorescent Zn^{2+} or Ca^{2+} indicators to both Zn^{2+} and Ca^{2+}. The representative probes included in the experiments were Newport Green, FluoZin-3, and Zinpyr-4 for Zn^{2+}, and Calcium Green-1, Fura-2, and Fluo-3 for Ca^{2+}.

2. Materials and methods

2.1. Fluorescence microscopy

Experiments were performed on a Zeiss Axiovert LSM510 confocal microscope. Dyes Calcium Green-1, Fluo-3, Newport Green, FluoZin-3 and Zinpyr-4 were excited with an Argon laser (488 nm) wavelength. The emission wavelength was set at 510 nm. Fura-2 was excited with dual wavelengths of 340 and 380 nm from the Enterprise laser for ratiometric imaging. All solutions were made with double de-ionized water. Dye solutions were made in either 100 mM KCl in 20 mM MOPS buffer adjusted to pH of 7.2, or in artificial cerebral-spinal fluid (ACSF) consisting of 121 mM NaCl, 1.75 mM KCl, 26 mM $NaHCO_3$, 10 mM Dextrose, and 1.25 mM KH_2PO_4 adjusted to a pH of 7.4. Ca^{2+} and Zn^{2+} were included in accordance with conditions of each test. All experiments were performed at room temperature. Dyes were used as 1 μM solutions, made from 1 mM stock solutions. The dyes used were cell impermeant salts of Newport Green DCF [(dipotassium salt, $K_D = 1$ μM) not Newport Green PDX ($K_D = 40$ μM)], Calcium Green-1, Fura-2, Fluo-3, FluoZin-3 (Molecular Probes, Eugene, Oregon) and Zinpyr-4 (NeuroBioTex, Galveston, Texas). In each experiment, increasing concentrations of Zn^{2+} were added to ACSF that already contained a fluorescent probe. Fresh saline was made before each experiment and excluded divalent ions.

Two scans were taken to establish a baseline prior to addition of Zn^{2+} (zinc chloride) or Ca^{2+} (calcium chloride). Images were scanned using a time series after each increasing concentration of solution was added. A Z-stack scan was performed prior to the addition of ion solution to find the optimal range of fluorescence for testing and scanning. Before each experiment the fluorescence intensity was set to an arbitrary unit of approximately '20' by adjusting the Detector gain (sensitivity) and the Amplifier offset (black level), so that graphs could be easily compared.

Care was taken to minimize potential Zn^{2+} contamination that might bring in background fluorescence and false negative in fluorescence detection. All solutions were kept in Teflon bottles, an upright confocal microscope was used to avoid using glass cover slips (attached to the bottom of cell culture dishes), and samples were transferred with metal-free pipette tips. The testing buffer/solution was made with *puriss* grade salts (Sigma or Fluka) and then further stripped of divalent metal ions by treatment with Chelex-100 beads (5%) in some experiments (Bio-Rad, Richmond, CA). Chemicals were transferred with Spatulas coated with tygon or polymethylpentene (Nalgene).

2.2. Fluorometric assays

Fluorescence responses of each dye were also analyzed with an F-2000 Fluorescence Spectrophotometer. ACSF and MOPS buffers were made using the same procedures as with the confocal microscopy. The same ion solutions were

made as with the confocal experiments. All experiments were performed at room temperature. The concentration of the dye solutions used was 100 or 500 nM made from a 1 mM stock. Newport Green and Calcium Green-1 were tested at 495 nm for excitation and 520 nm for emission. Zinpyr-4 and FluoZin-3 were tested at 488 nm for excitation and 520 nm for emission. Fura-2 was tested at excitations of 340 and 380 nm and an emission of 510 nm. For each trial, 2 mL of the dye solution was placed in a cuvette and a stirring bead was added and then the cuvette was placed in the spectrofluorometer. A 30 s scan was run to establish a constant baseline value and then increasing concentrations of Zn^{2+} or Ca^{2+} (10 nM up to 1 mM) were added to the cuvette.

2.3. Hippocampal slice preparation

Male Sprague–Dawley rats weighing approximately 200–300 g were used as tissue donors for the experiments. Rats were anesthetized with Ketamine (0.1 mL per 100 g). The anesthetized rats were decapitated and the brain tissue is removed quickly and placed in ice-cold (1–4 °C) artificial cerebral-spinal fluid. Tissue slices of the hippocampal region were cut with a Vibratome at approximately 200–250 μm in thickness. This procedure is performed in an artificial cerebral-spinal fluid with a composition of 121 mM NaCl, 1.75 mM KCl, 26 mM $NaHCO_3$, 10 mM Dextrose, 1.25 mM KH_2PO_4, 1.3 mM $MgCl_2$, and 2.4 mM $CaCl_2$ continuously bubbled with 95% O_2/5% CO_2. The slices were preloaded in 20 μM membrane-permeant Calcium Green-1 AM (Molecular Probes), 0.1% pluronic acid, and 0.5% dimethyl sulfoxide for 1 h [33]. TPEN, a Zn^{2+} chelator, did not change the background fluorescence in pyramidal cells, consistent with the fact that there is little free $[Zn^{2+}]_i$ in the resting state [30]. During simulated ischemia experiments, hippocampus slices were bathed with ACSF lacking glucose (NaCl adjusted to 131 mM) and perfused with 95% N_2 and 5% CO_2 for 30 min in the oxygen and glucose deprived (OGD) ACSF, followed by reperfusion in normal ACSF.

3. Results

3.1. In vitro responses of fluorescent Zn^{2+} indicators to Zn^{2+} and Ca^{2+}

Properties such as the selectivity and sensitivity (range of detection) of a metallofluorescent probe dictate the outcome of ion measurement. We first evaluated the response of fluorescent Zn^{2+} probes in ACSF to different concentrations of Zn^{2+} (Fig. 1A–C). Commercial data regarding these fluorescent probes are almost always obtained in spectrofluorometer-based assays. Although *in vitro* spectra are available for these fluorescent probes [31,32], it cannot be assumed that dyes will show identical ion-binding properties under all test conditions (i.e. particular equipment, different pH, concentrations of particular ions, overall ionic strength, and so forth). Therefore, we tested the ability of the fluorescent indicators to measure Ca^{2+} or Zn^{2+} under conditions similar to those used in the tissue preparation of interest, so that the results will have direct relevance to our *in situ* experiments.

The representative Zn^{2+} probes used in these experiments are Newport Green DCF, FluoZin-3, and Zinpyr-4. Of these, Newport Green has a relatively low affinity for binding to Zn^{2+} (higher K_D) whereas Zinpyr-4 and FluoZin-3 have a relatively high affinity for binding to Zn^{2+} (smaller K_D). Addition of 1 nM Zn^{2+} yielded no change in fluorescence of any indicator tested in this study. It could be as a result of Zn^{2+} contamination (see Section 2) which contributed to background fluorescence. Fig. 1A shows that the response of FluoZin-3 (K_D for Zn^{2+} = 15 nM, Molecular Probes) to Zn^{2+} reaches a plateau, with the probe becoming saturated at about 1 μM of Zn^{2+}. Another high affinity probe, Zinpyr-4 (K_D for Zn^{2+} = 1.0 nM, NeuroBioTex), was saturated at about 10 μM Zn^{2+} (Fig. 1B). These new generations of Zn^{2+} probes generate very bright fluorescence when bound to Zn^{2+}; their fluorescence intensity at 1 μM Zn^{2+} was several times greater than that detected from 0 to 100 nM Zn^{2+}. In contrast, the low affinity probe Newport Green (K_D for Zn^{2+} = 1–3 μM, [31,33,34]) did not become saturated even at concentrations up to 300–500 μM Zn^{2+} (Fig. 1C). Visible precipitation of $Zn(OH)_2$ became apparent as the Zn^{2+} concentration reached 500 μM or 1 mM, eventually quenching the fluorescence. Thus, the precise Zn^{2+} concentration at which Newport Green becomes saturated is unclear. However, the effective Zn^{2+} detection range of Newport Green was about 1 μM–1 mM Zn^{2+}, whereas the range of Zn^{2+} detection of FluoZin-3 and Zinpyr-4 were 10 nM–1 μM and 100 nM–10 μM Zn^{2+}, respectively. Because commercial data have been obtained in MOPS solution [31,35], we repeated the same tests in MOPS-KCl solution (data not shown). No significant difference was observed when comparing results from the two different salines.

Competitive binding of other cations, namely Ca^{2+}, complicates measuring Zn^{2+} in cells or environmental samples with fluorescent indicators originally designed for detection of Ca^{2+}. The sensitivity of Newport Green to Ca^{2+} has been previously published [33,36], however, the sensitivity of FluoZin-3 and Zinpyr-4 to Ca^{2+} has not been explicitly reported. Fig. 1D–F shows the responses of these fluorescent Zn^{2+} probes to Ca^{2+}. Of all of these probes, FluoZin-3 showed the greatest Ca^{2+} sensitivity at concentrations between 1 μM and 1 mM. The peak value of fluorescence intensity of FluoZin-3 in response to Ca^{2+} was about 20–30% of the maximum value in response to Zn^{2+} (Fig. 1A and D). Indeed, we initially observed the Ca^{2+} sensitivity of FluoZin-3 when we noticed bright green background fluorescence in ACSF containing 2.5 mM Ca^{2+}. However, the response of FluoZin-3 to Ca^{2+} at concentrations below 100 μM was negligible, because it was less than 5% of the maximum fluorescence to Zn^{2+} at 1 μM (Fig. 1A and D). The other

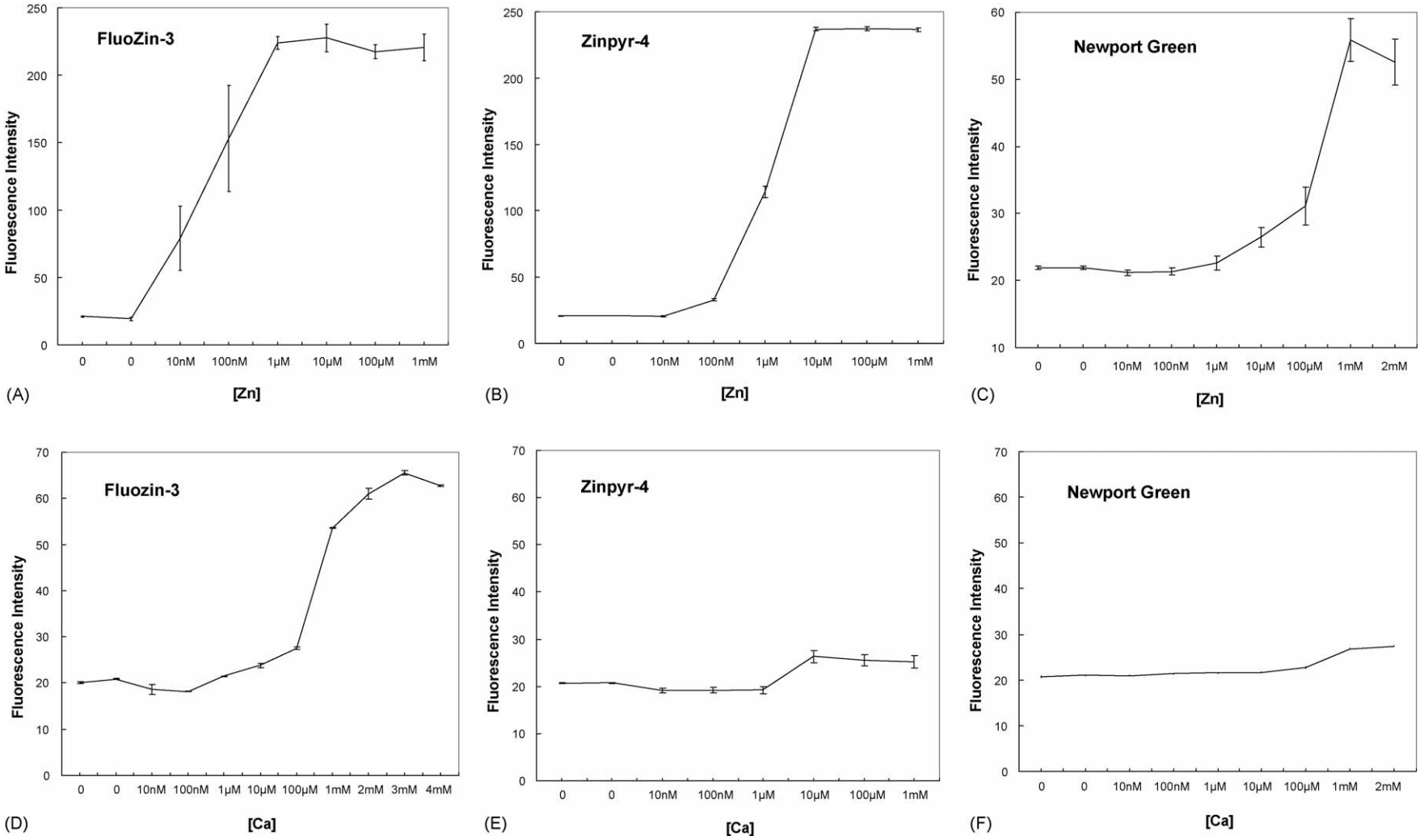

Fig. 1. Selectivity and sensitivity of fluorescent Zn^{2+} indicators to Zn^{2+} and Ca^{2+}. (A–C) are concentration–response curves to Zn^{2+} for FluoZin-3, Zinpyr-4, and Newport Green. Newport Green had the widest range of detection for Zn^{2+}, whereas FluoZin-3 and Zinpyr-4 have a much smaller window of detection to Zn^{2+}. (D–F) are concentration–response curves to Ca^{2+} of the same Zn^{2+} indicators. Of all these probes, FluoZin-3 showed the largest response to Ca^{2+}. All data were obtained through fluorescence microscopy. All graphs plot mean ± S.E.; $n = 3$–6 different experiments for each value.

two Zn^{2+} probes showed little sensitivity to Ca^{2+}. Unlike FluoZin-3, which detected Ca^{2+} over a broad range of concentrations (1–1000 μM), Zinpyr-4 and Newport Green did not exhibit substantial increases in fluorescence with increasing Ca^{2+} concentration. The responses of Zinpyr-4 and Newport Green to Ca^{2+} were minimal, with a small response at about 10 μM Ca^{2+} in Zinpyr-4 and at about 1 mM Ca^{2+} in Newport Green. The same tests were performed separately in ACSF and MOPS-KCl solutions. We did not find significant differences in the data collected in the two solutions. Mg^{2+} sensitivity was also examined. None of above probes showed sensitivity to Mg^{2+} at test concentrations up to 2 mM (data not shown).

3.2. *In vitro responses of fluorescent Ca^{2+} probes to Zn^{2+}*

The Zn^{2+} sensitivity of fluorescent Ca^{2+} probes such as Fura-2 has previously been reported in detail [28,29]. However, the information concerning Zn^{2+} sensitivity has largely been ignored, and it has not been explicitly studied in a physiological system. Fig. 2 shows the responses in ACSF of the most commonly used Ca^{2+} fluorescence indicators, Calcium Green-1, Fura-2, and Fluo-3, to different concentrations of Zn^{2+} and Ca^{2+}. All three probes showed an affinity for both Ca^{2+} and Zn^{2+} and the response curves for both divalent ions were very similar. All probes showed similar ranges of detection for both Ca^{2+} and Zn^{2+}. Fura-2 (Fig. 2C) was excited at 340 nm with a fluorescence spectrophotometer because its excitation wavelengths were ill suited to laser excitation. The fluorescence of Calcium Green-1 and Fura-2 in response to Zn^{2+} was slightly higher than their fluorescence in response to Ca^{2+}. This is consistent with previous reports that Fura-2 is more sensitive to Zn^{2+} than to Ca^{2+} because of its tighter binding [29]. These data suggest that Zn^{2+} could contribute to responses measured with Ca^{2+} sensitive-dyes that are believed to derive from Ca^{2+} alone.

3.3. *Calcium Green-1 detected incremental increases in Zn^{2+} concentration in the presence of physiological levels of Ca^{2+}*

Fluorescent Ca^{2+} probes are sensitive to Zn^{2+}; therefore, the presence of Zn^{2+} in a biological system could interfere with Ca^{2+} determination. Conversely, Ca^{2+} could interfere with Zn^{2+} determination, particularly in a Ca^{2+}-rich environment, i.e. biological Zn^{2+} measurement by Ca^{2+} probes could be quelled by normal concentrations of Ca^{2+}. Although most Ca^{2+} indicators have a higher affinity for Zn^{2+} than for Ca^{2+}, it has been assumed that the presence of physiologically relevant Ca^{2+} in the sample will prevent Zn^{2+} from binding to fluorescent Ca^{2+} indicators, possibly by surrounding the indicator with a sufficient number of calcium ions. Another indication of Zn^{2+} selectivity over Ca^{2+} would be measurement of Zn^{2+} responses in the presence of a relatively high Ca^{2+} concentration, in which the probe is saturated with Ca^{2+} prior to the addition of Zn^{2+}. Calcium Green-1 was used to test this selectivity. Fig. 3A shows that Calcium Green-1

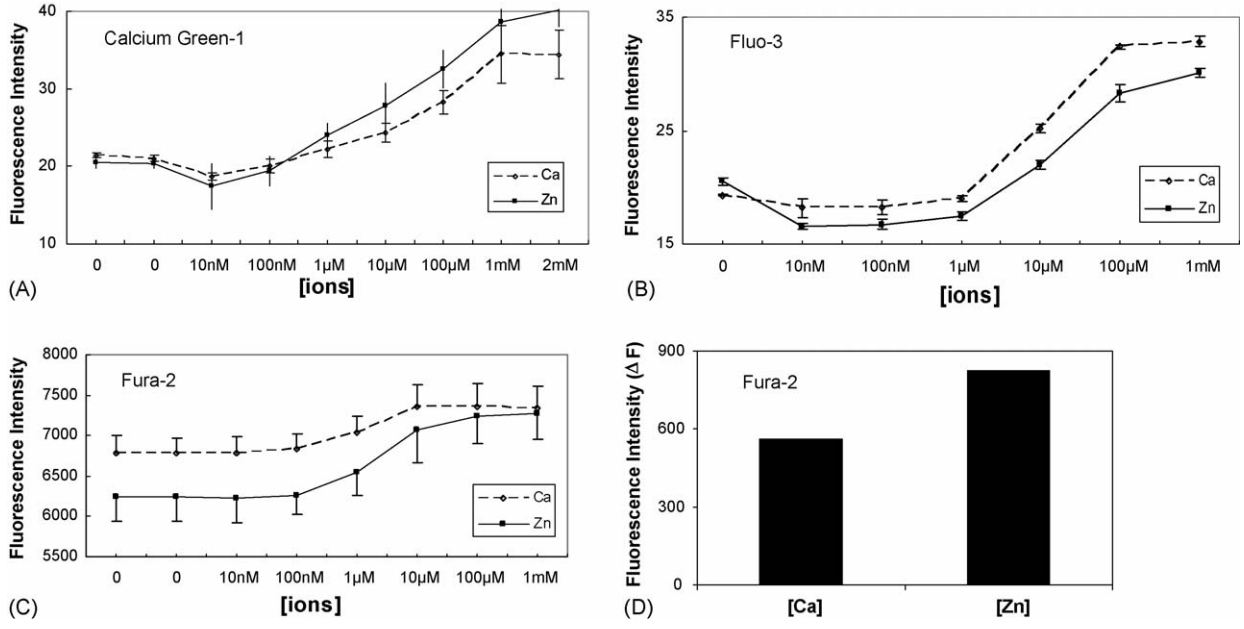

Fig. 2. Selectivity and sensitivity of fluorescent Ca^{2+} indicators. (A) Concentration–response curves of Calcium Green-1 for Zn^{2+} and Ca^{2+}. (B) Concentration–response curves of Fluo-3 for Zn^{2+} and Ca^{2+}. (C) Concentration–response curves of Fura-2 to Zn^{2+} and Ca^{2+}. All three probes had an affinity for both Ca^{2+} and Zn^{2+} and the response ranges of both divalent ions were very similar. To compare the difference in sensitivity of Fura-2 to Zn^{2+} and Ca^{2+}, values acquired separately at 1 μM Zn^{2+} and Ca^{2+} in C were extracted and re-plotted in (D). Data for Calcium Green and Fluo-3 were obtained through fluorescence microscopy; data for Fura-2 were obtained through spectrofluorometry. All graphs plotted are mean ± S.E.; $n = 3$–6 different experiments for each value.

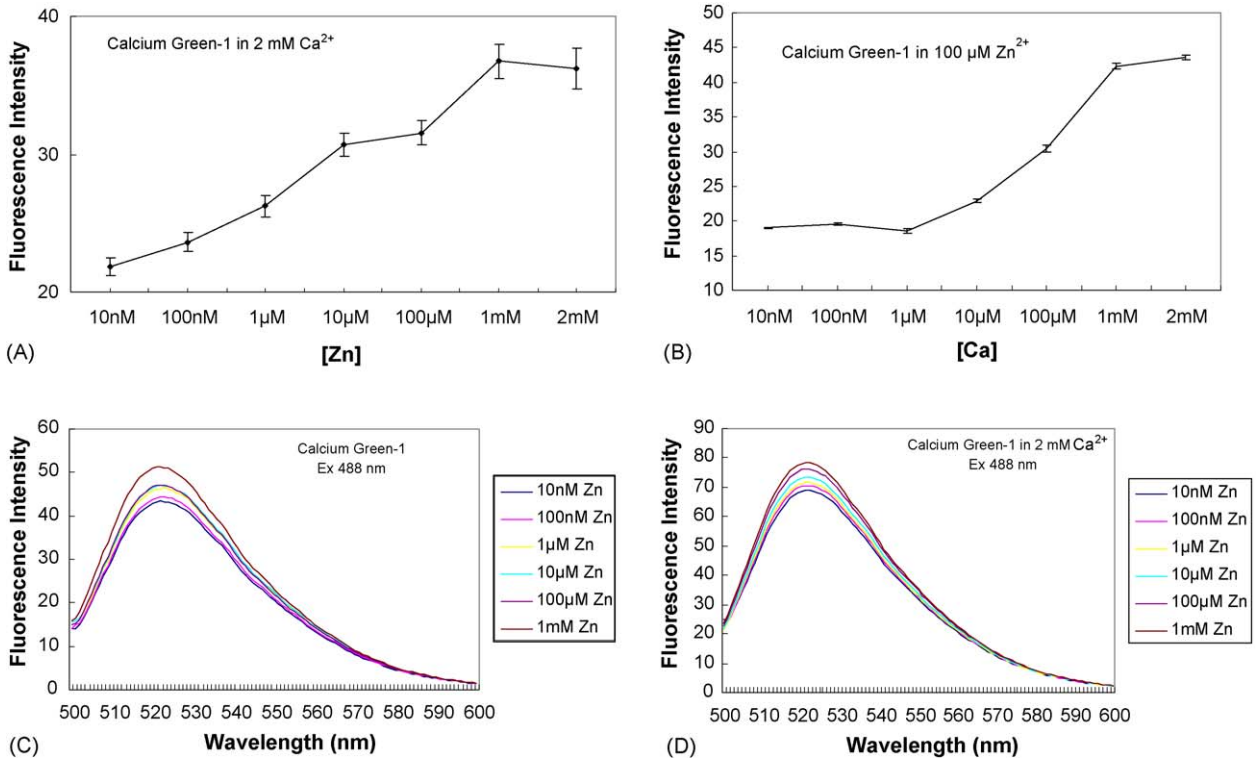

Fig. 3. The response of Calcium Green-1 to Zn^{2+} in the presence of 2 mM Ca^{2+} and to Ca^{2+} in the presence of 100 μM Zn^{2+}. (A) The concentration–response curve of Calcium Green-1 to Zn^{2+} in the presence of 2 mM Ca^{2+}. The baseline was set after the addition of Ca^{2+}. (B) The concentration–response curve of Calcium Green-1 to Ca^{2+} in the presence of 100 μM Zn^{2+}. The baseline was set after the addition of Zn^{2+}. Graphs plotted are mean ± S.E.; $n = 3$ different experiments for each point. (C and D) Graphs of spectroscopic responses of Calcium Green-1 to increasing concentration of Zn^{2+} in MOPS medium. Measurements were made in the absence (C) or in the presence (D) of 2 mM Ca^{2+}.

still detected Zn^{2+} in the presence of up to 2 mM Ca^{2+}. In these experiments the baseline was set after the addition of Ca^{2+}. There was no difference in the concentration–response relationship of Zn^{2+} obtained with or without Ca^{2+}. Furthermore, the presence or absence of Ca^{2+} had no significant effect on the spectroscopic responses of Calcium Green-1 to Zn^{2+} (Fig. 3C and D). The optimal emission wavelength for Zn^{2+} at 520 nm in the absence of Ca^{2+} was not changed by the presence of 2 mM Ca^{2+}. Although the mean fluorescence intensity of Calcium Green-1 to Zn^{2+} in the presence of 2 mM Ca^{2+} was greater than that observed in the absence of Ca^{2+}, the difference was not significant and was essentially within experimental error. Similar results were obtained for Fura-2 with the fluorescence spectrophotometer (data not shown here). We also tested the fluorescent responses of Calcium Green-1 to Ca^{2+} in the presence of Zn^{2+}. In these experiments baseline was also re-adjusted before adding Ca^{2+} to cancel the background fluorescence caused by Zn^{2+}. Fig. 3B shows the sensitivity of the Ca^{2+} probe to Ca^{2+} in the presence of 100 μM Zn^{2+} with no difference observed in the fluorescence response to Ca^{2+}. Our results were not in agreement with the assumption that the presence of physiologically relevant Ca^{2+} in the sample prevents Zn^{2+} from binding to fluorescent indicators. The data instead suggests that either Zn^{2+} can still bind with Calcium Green-1 in a concentration independent manner, or that Zn^{2+} does not compete with Ca^{2+} for the same binding site; rather Zn^{2+} may bind at a site other than the Ca^{2+} site.

Next, we evaluated the fluorescent responses of Calcium Green-1 to Zn^{2+} or Ca^{2+} under conditions in which alternating increments in Zn^{2+} and Ca^{2+} concentrations were added (Fig. 4). Zn^{2+} at 10 nM–100 μM increases the fluorescence intensity of Calcium Green-1. Then, 100 μM Ca^{2+} was added into the same medium with 100 μM Zn^{2+} already present and induced a further increase in fluorescence. Zn^{2+} at 1 mM produced an additional increase after the addition of 100 μM Ca^{2+}, and 1 mM Ca^{2+} induced a further increase before Calcium Green-1 was saturated by 2 mM Ca^{2+} (in the presence of 1 mM Zn^{2+}). The ion-metal response of the probe to Zn^{2+} and Ca^{2+} seem to complement each other, because the overall fluorescence intensities were higher in competition tests than that in individual tests. There was no change in the range of the detection or sensitivity of Calcium Green-1 to either ion. This is the most compelling evidence that Zn^{2+} and Ca^{2+} complement each other or they bind to independent sites in Calcium Green-1.

3.4. Fluorescent Ca^{2+} probe detecting cellular Zn^{2+} in rat brain slice

Our results raise two important issues. First, we want to study whether a commonly used fluorescent Ca^{2+} indicator,

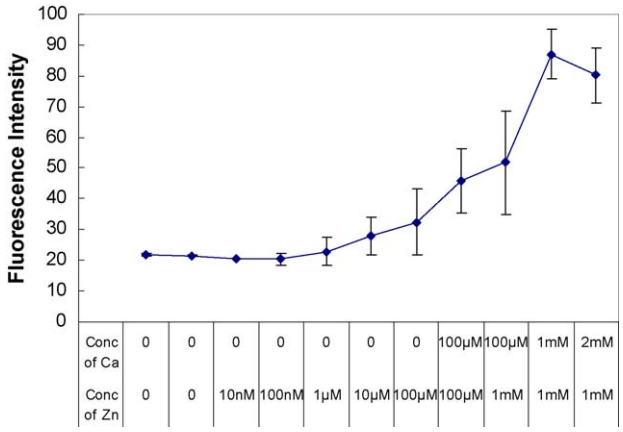

Fig. 4. Competition experiment of Calcium Green-1 with Ca^{2+} and Zn^{2+}. Additional increments of Zn^{2+} and Ca^{2+} both produced increased fluorescence even in the presence of the other ion. The fluorescence intensity increased when the Zn^{2+} concentration was incrementally increased to 100 μM. The fluorescence intensity was further increased with the addition of 100 μM Ca^{2+}. One micromolar Zn^{2+} added to the same medium induced a further increase in the Calcium Green-1 response after addition of 100 μM Ca^{2+}. The values plotted are the mean ± S.E.; $n = 4$ for each point. Conc of Ca, concentration of Ca^{2+}; Conc of Zn, concentration of Zn^{2+}.

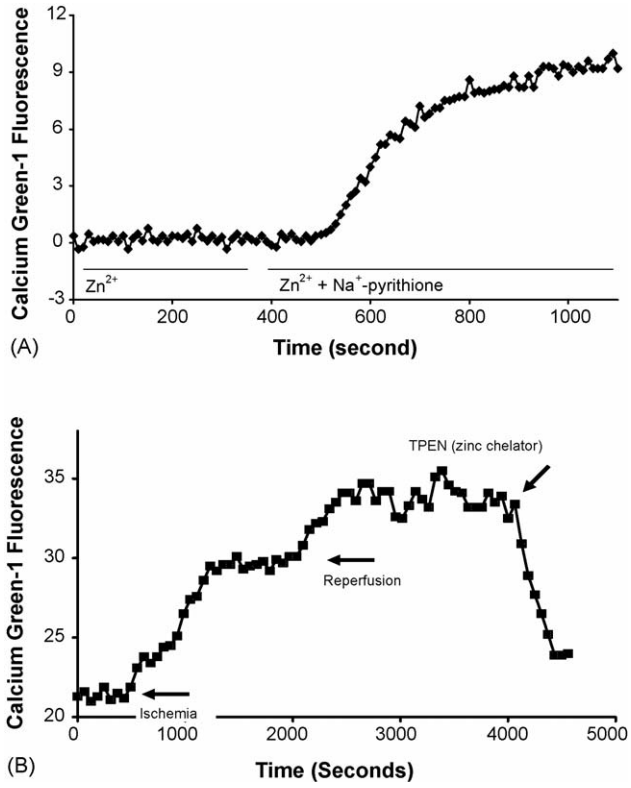

Fig. 5. Detecting cellular Zn^{2+} with membrane permeable Calcium Green-1 AM in rat hippocampal slices. (A) Five-minute exposure to 300 μM Zn^{2+} produced no change in Calcium Green-1 fluorescence. Subsequent addition of 100 μM Na^+ pyrithione, a Zn^{2+} ionophore, with Zn^{2+} resulted in a progressive increase in fluorescence. No Ca^{2+} was added in the perfusate. (B) Oxygen and glucose deprivation (OGD) resulted in a progressive increase in Calcium Green-1 fluorescence that was reduced by the addition of 100 μM TPEN.

such as Calcium Green-1, can detect Zn^{2+} *in situ*. This was investigated in pyramidal cells of freshly cultured rat brain hippocampal slices. In the experiment shown in Fig. 5A, slices were pre-loaded with 20 μM Calcium Green-1 and then placed in a recording chamber on the microscope stage with continuous perfusion of biological saline (ACSF without added Ca^{2+}). To investigate whether Zn^{2+} can be detected *in situ* with a fluorescent Ca^{2+} probe, 300 μM Zn^{2+} and 100 μM Na-pyrithione, a Zn^{2+} ionophore that facilitates ion influx into cells, were added to the perfusate. In the presence of Zn^{2+} alone we observed minimal change of intracellular fluorescence of Calcium Green-1. Pyrithione alone without Zn^{2+} did not cause any increase in fluorescence (data not shown). Increases in $[Zn^{2+}]_i$ were detected when both Zn^{2+} and pyrithione were present, and the fluorescence signal was reversed by the addition of TPEN. This result indicates that the influx of Zn^{2+} and a rise in $[Zn^{2+}]_i$ change the fluorescent intensity of Calcium Green-1 and demonstrates that these fluorescent Ca^{2+} indicators can effectively measure the dynamic change of cytosolic Zn^{2+}.

The next question one may ask is whether Zn^{2+} transients could contribute to Ca^{2+} transients conventionally detected with fluorescent Ca^{2+} probes. This question has become logical nowadays since growing evidence has demonstrated an increase of $[Zn^{2+}]_i$ in various neural activities [11]. To test this speculation, we pre-loaded hippocampal slices with Calcium Green-1 AM (Fig. 5B). We then depolarized the pyramidal cells under conditions of oxygen and glucose deprivation (OGD). Many studies have indicated an increasing $[Ca^{2+}]_i$ (Ca^{2+} overload) following OGD. However, there are also reports showing increases in $[Zn^{2+}]_i$ under OGD [37,38]. The representative trace in Fig. 5B shows that pyramidal cells responded to stimulation with OGD with enhanced Calcium Green-1 fluorescence. However, the peak of Calcium Green-1 transients were reduced in the presence of TPEN (100 μM), suggesting that Zn^{2+} contributes to the rise of Calcium Green-1 fluorescence intensity in the hippocampal neurons.

4. Discussion

In this study, we investigated the responses of several representative Zn^{2+} and Ca^{2+} fluorescent dyes to both Ca^{2+} and Zn^{2+}. The *in vitro* experiments were carried out under conditions similar to those used with our tissue preparation so that the results would have direct relevance to the *in situ* experiments. Our results suggest that (1) Zn^{2+}-sensitive dyes are more specific for their targeted ion than Ca^{2+}-sensitive dyes, showing very little interaction with Ca^{2+}, whereas Ca^{2+}-sensitive dyes are less specific for their targeted ion, showing a wide range of interaction with Zn^{2+}. (2) Fluorescent Ca^{2+} indicators respond to Zn^{2+} with increased fluorescence at increased Zn^{2+} concentrations in the presence of a physiologically relevant concentration of Ca^{2+}. This is not consistent with the assumption that a high concentration of Ca^{2+} prevents Zn^{2+} from binding to fluorescent Ca^{2+}-indicators. (3)

Zn^{2+} binding site(s) may differ from the site(s) that Ca^{2+} occupies in Ca^{2+} sensitive dyes. (4) Furthermore, our data demonstrate that fluorescent Ca^{2+} indicators such as Calcium Green-1 can be used effectively to measure dynamic change of cytosolic Zn^{2+} *in situ*. (5) Finally, our results suggest that Zn^{2+} signals detected with Ca^{2+} probes may contribute to conventional Ca^{2+} signals in a biological system. One implication of these results is that data obtained using Ca^{2+}-sensitive dyes may need to be re-examined to determine if previously acquired results could, in part, be due to Zn^{2+}.

4.1. Fluorescent Zn^{2+} probes

While there are many roles for Zn^{2+} in the brain and other organ systems, the key to understanding and studying these roles is the ability of fluorescent indicators to bind to and detect Zn^{2+} under physiological and pathophysiological conditions. Selectivity and sensitivity are two basic components that affect Zn^{2+} detection by a probe. Several new Zn^{2+} probes have been developed and marketed in recent years. There is little literature available describing the properties of Zn^{2+} probes, especially their properties in biological systems. It is thus critical to be aware of the specificity and selectivity of a probe to a targeted ion in the presence of potential interferent(s). Values for the selectivity and sensitivity of a specific probe, which affect the outcome of experimental results and the interpretation of data, must be examined before its application in any given experimental system.

4.1.1. Selectivity (specificity)

Although several of them are still EDTA derivatives, the new generation of Zn^{2+} probes are more selective than earlier probes; selectivity has become less of a concern with fluorescent Zn^{2+} probes as most of them are highly selective for Zn^{2+} over Ca^{2+} [31]. All three of the tested probes have been reported to be insensitive to physiological levels of Ca^{2+} [33–36]. We showed that the response of Zinpyr-4 and Newport Green to Ca^{2+} was limited and that these dyes did not exhibit substantial increases in fluorescence with increasing Ca^{2+} concentration. FluoZin-3 showed the largest response to Ca^{2+}, which was about 20–30% of the maximum value observed when FluoZin-3 was used to detect Zn^{2+} (Fig. 1). The ability of FluoZin-3 to bind Zn^{2+} may be affected by the ability of this fluorophore to bind Ca^{2+}. According to Molecular Probes [31], the Zn^{2+}-binding affinity of FluoZin-3 is perturbed by Ca^{2+} starting at around 1–5 μM Ca^{2+} [31]. Indeed, the Ca^{2+} sensitivity of FluoZin-3 is comparable to its sensitivity to Zn^{2+} (Figures 19.76 and 19.78 in [31]). We initially noticed the Ca^{2+} sensitivity of FluoZin-3 when we used it to detect Zn^{2+} in an acute brain slice experiment in which we observed bright background green fluorescence in physiological medium (ACSF) containing 2.5 mM Ca^{2+}. Biological Zn^{2+} measurements may be complicated by the presence of Ca^{2+}. Therefore, a probe like FluoZin-3 is not an optimal choice for probing Zn^{2+} in an environment in which high concentrations of Ca^{2+} are present, such as the extracellular space. *However*, in our studies, the responses of FluoZin-3 to Ca^{2+} concentrations below 100 μM was negligible, being less than 5% of the maximum response to Zn^{2+} at 1 μM (Fig. 1). FluoZin-3 (and also Zinpyr-4) appears to be very sensitive to low concentrations of Zn^{2+}. There was a wide span of fluorescence intensity over Zn^{2+} concentrations ranging from 100 nM to 1 μM. Taken together, we propose that FluoZin-3 and Zinpyr-4 are the best choices for probing Zn^{2+} in an environment such as intracellular space where Ca^{2+} concentration reportedly is below 100 μM.

4.1.2. Sensitivity (capacity)

The rule of thumb for capacity is that a probe, at concentrations of probe close to its K_D, can generally detect concentrations of its target up to 1-2 order of its K_D. This is consistent with the results in this study, although every probe detected Zn^{2+} over a different range. Newport Green (Zn^{2+} K_D = 1 μM) showed a response to Zn^{2+} over the range of 1 μM to 1mM without becoming saturated. FluoZin-3 (Zn^{2+} K_D = 15 nM) and Zinpyr-4 (Zn^{2+} K_D = 1.0 nM) were saturated at relatively low concentrations of Zn^{2+} (1 and 10 μM, respectively). The inability of FluoZin-3 and Zinpyr-4 to detect Zn^{2+} at low μM concentrations limits their application in biological systems where the background Zn^{2+} level is close to the concentration at which they become saturated.

4.2. Fluorescent Ca^{2+} probes

In this study all three Ca^{2+} probes showed an affinity for both Ca^{2+} and Zn^{2+} and the response curves for both divalent ions were very similar (Fig. 2). Although nearly all fluorescent Ca^{2+} indicators, in fact, also bind Zn^{2+} with high affinity [29], their sensitivity to Zn^{2+} and interference by the presence of Zn^{2+} have been largely ignored. This may be because Zn^{2+} has been regarded as merely one of the trace elements in biological systems. The evidence of increases in intracellular Zn^{2+} during neural activities poses a dilemma: elevation of intracellular Zn^{2+} could comprise part of the conventionally measured intracellular Ca^{2+} signals.

4.2.1. Calcium Green-1 is able to detect incremental changes in $[Zn^{2+}]$ in the presence of physiological levels of Ca^{2+}

It has been assumed that the presence of physiologically relevant Ca^{2+} in the sample will prevent Zn^{2+} from binding to fluorescent indicators, possibly by surrounding the indicator with a sufficient number of calcium ions. Physiological levels of Ca^{2+} have been expected to quell biological Zn^{2+} signals by Ca^{2+} probes. For example, extracellular Ca^{2+} is about 2 mM; any increased $[Zn^{2+}]$ (possibly in nM and μM range) in the extracellular space would not be expected to compete with this 'mighty' Ca^{2+}. However, our results (Fig. 3) were not consistent with this assumption. The fluorescent Ca^{2+} probe Calcium Green-1 was still capable of detecting Zn^{2+}

in the presence of a physiologically relevant concentration of Ca^{2+}, up to 2 mM. There was no difference in the relationship of Zn^{2+} concentration to fluorescent response obtained with or without Ca^{2+}. The mean fluorescence intensity of Calcium Green-1 to Zn^{2+} in the presence of 2 mM Ca^{2+} was slightly (not significantly) greater than that observed in the absence of Ca^{2+}. Since the baseline was re-adjusted before adding Zn^{2+} to cancel the background fluorescence caused by Ca^{2+}, this might be the (facilitating) effect of Ca^{2+} present on the responses to Zn^{2+}. Similar results were obtained for the ratiometric indicator Fura-2 with the fluorescence spectrophotometer. Not only are these fluorescent Ca^{2+} probes sensitive to Zn^{2+}, but the presence of Ca^{2+} minimally disturbs their response to Zn^{2+}. They are effective Zn^{2+} indicators.

The metal-ion response of the probe to Zn^{2+} and Ca^{2+} seem to complement each other, because the overall fluorescence intensities were higher in competition tests than those in individual tests. This observation could be explained by that, for example, the probe-Zn^{2+} complexes produce brighter fluorescence when Zn^{2+} and Ca^{2+} competed for the binding site. On the other hand, Ca^{2+} and Zn^{2+} do not perturb each other's response. 2 mM Ca^{2+} does not interfere with the measurement of Zn^{2+} by Calcium Green-1 nor does 100 μM Zn^{2+} disturb the sensitivity of this probe to Ca^{2+} (Fig. 3A and B). It seems that the mechanism of Zn^{2+} binding does not involve competing with Ca^{2+} for the same binding site, rather that Zn^{2+} may bind at a site different from the Ca^{2+} site. If Ca^{2+} and Zn^{2+} compete for the same binding site on Calcium Green-1 then one would expect not to see a change in fluorescence as Zn^{2+} is added to a Ca^{2+}/Calcium Green-1 solution. More experimentation or molecular modeling is needed to understand the binding property of this fluorescent probe.

4.2.2. Zn^{2+} responses contribute to the "Ca^{2+} response" conventionally detected with Calcium Green-1 in biological systems

We suspected that the sensitivity of Ca^{2+} probes to Zn^{2+} could distort their detection of Ca^{2+}. Our data (Fig. 5) suggest that Zn^{2+} contributes to responses measured with Ca^{2+}-sensitive dyes that have been believed to result from Ca^{2+} alone. The metallothioneins can serve as intracellular reservoirs for release of Zn^{2+} during signaling or during deficiency states. Although zinc lacks redox activity, Zn^{2+}-dependent cytotoxicity may occur when Zn^{2+} is released from zinc-binding proteins of the perikaryal cytoplasm of the cell and then from mitochondrial stores under oxidative stress [10,19,20]. Like calcium, excess free zinc in body tissues is toxic. Recently, evidence from our group and others groups suggested that the reactive free radical species nitric oxide (NO) may be involved in the regulation of Zn^{2+} homeostasis [10,19,37,39]. NO may cause Zn^{2+} release from metallothionein and zinc-finger proteins by *S*-nitrosylation of cysteine clusters that bind Zn^{2+} [40–42]. Zn^{2+} can be also released from a specific subset of glutamatergic nerve terminals throughout the mammalian cortex of the brain. The released Zn^{2+} then translocates into the post-synaptic neuron through Zn^{2+} permeable channels [11,33], and creates a $[Zn^{2+}]_i$ transient. Thus, Zn^{2+} signals are likely present under numerous circumstances.

The key conclusion here is that commonly used fluorescent Ca^{2+} indicators can effectively perceive Zn^{2+} independently of Ca^{2+}. Much of our current knowledge of Ca^{2+} as a second messenger comes from its binding to fluorescent indicators. Given the results presented in this paper, we are convinced that more selective Ca^{2+} probes are needed to define Ca^{2+} signaling. We may need to reconsider the role of Ca^{2+} under conditions in which this was determined based on detection of Ca^{2+} by fluorescent indicators. This question has become logical currently because mounting evidence demonstrates an increase in $[Zn^{2+}]_i$ in neural activities. Furthermore, possible interactions between Ca^{2+} and Zn^{2+} must be understood if both play important roles in cellular signaling.

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