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Fluorescence imaging study of extracellular zinc at the hippocampal mossy fiber synapse

Chinthasagar Bastian, Yang V. Li *

Department of Biomedical Science, Program in Biological Sciences, Ohio University, Athens, OH 45701, USA

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Abstract

Although synaptically released, vesicular Zn^{2+} has been proposed to play a neuromodulatory or neuronal signaling role at the mossy fiber-CA3 synapse, Zn^{2+} release remains controversial, especially when detected using fluorescent imaging. In the present study, we investigated synaptically released Zn^{2+} at the mossy fiber (MF) synapse in rat hippocampal slices using three chemically distinct, fluorescent Zn^{2+} indicators. The indicators employed for this study were cell membrane impermeable (or extracellular) Newport Green $K_{DZn^{2+}} \sim 1\,\mu M$, Zinpyr-4 $K_{DZn^{2+}} \sim 1\,nM$ and FluoZin-3 $K_{DZn^{2+}} \sim 15\,nM$, chosen, in part, for their distinct dissociation constants. Among the three indicators, FluoZin-3 was also sensitive to Ca^{2+} $K_{DCa^{2+}} \sim 200$–$300\,\mu M$ which was present in the extracellular medium ($[Ca^{2+}]_o > 2\,mM$). Hippocampal slices loaded with either Newport Green or FluoZin-3 showed increases in fluorescence after electrical stimulation of the mossy fiber pathway. These results are consistent with previous studies suggesting the presence of synaptically released Zn^{2+} in the extracellular space during neuronal activities; however, the rise in FluoZin-3 fluorescence observed was complicated by the data that the addition of exogenous Zn^{2+} onto FluoZin-3 loaded slices gave little change in fluorescence. In the slices loaded with the high-affinity indicator Zinpyr-4, there was little change in fluorescence after mossy fiber activation by electrical stimulation. Further study revealed that the sensitivity of Zinpyr-4 was mitigated by saturation with Zn^{2+} contamination from the slice. These data suggest that the sensitivity and selectivity of a probe may affect individual outcomes in a given experimental system.

Keywords: Zinc; Calcium; Fluorescence; Mossy fiber; Neurotransmitter; Synapse; FluoZin-3; Zinpyr-4; Newport Green

Zinc (Zn^{2+}) is found in a specific subset of glutamatergic nerve terminals throughout the brain cortex and limbic region and is especially abundant in hippocampal mossy fiber synapses of the hilar and CA3 regions [2]. Investigations on the role of Zn^{2+} in cellular processes has been facilitated by the recent development of Zn^{2+}-sensitive fluorometric probes, enabling the measurement of Zn^{2+} concentrations in both extracellular and intracellular environments [4]. Evidence showing the co-localization of Zn^{2+} with glutamate inside synaptic vesicles suggests the possibility that Zn^{2+} is released during exocytosis [2]. To observe Zn^{2+} release from neuronal terminals directly, it is necessary to employ a cell-impermeable fluorescence-based Zn^{2+} indicator. Zn^{2+} release during neuronal activity has been observed and characterized by means of fluorescence imaging [7,9,12]. Characterization of this Zn^{2+} release has revealed that it occurs in a fashion similar to neurotransmitters: the release is both Ca^{2+}-dependent and tetrodotoxin-sensitive [7]. However, such observations may sometimes be ill-informed as scant information exists to describe the properties of Zn^{2+} probes in biological systems. In addition, Zn^{2+} measurements in cells and environmental samples by fluorescent indicators originally designed to detect Ca^{2+} are further complicated by the competitive binding of other cations, namely Ca^{2+} itself [8]. Therefore, in this study, we applied three extracellular fluorescent Zn^{2+} indicators under the same experimental conditions to examine the extracellular Zn^{2+} induced by the stimulation in the mossy fiber (MF) pathway of the hippocampus.

Transverse hippocampal slices of thickness 200–250 μm from adult male Sprague Dawley rats were prepared using a Vibratome 3000 Plus Automated (St. Louis, MO). Each slice was equilibrated for 30 min prior to image acquisition. Perfusing solutions were prepared so that the osmolarity was 300 ± 5 mosm/kg and contained the following concentrations: 121 mM NaCl, 1.75 mM KCl, 26 mM $NaHCO_3$, 10 mM dextrose, 1.25 mM KH_2PO_4, 2.5 mM $CaCl_2{\cdot}2H_2O$ and 1.3 mM

* Corresponding author at: Department of Biomedical Science, Ohio University, 346 Irvine Hall, Athens, OH 45701, USA. Tel.: +1 740 593 2389.
E-mail address: li@oucom.ohiou.edu (Y.V. Li).

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$MgCl_2 \cdot 6H_2O$. All solutions were constantly bubbled with 95% O_2 and 5% CO_2. Slices were loaded with dye by incubation in ACSF with the given fluorescent indicator (10 μM) for 5 or 10 min. Optimal loading was confirmed by Z-series scanning. A bipolar, concentric electrode (World Precision Instruments, Inc., Sarasota, Florida) consisting of a polyimide insulated tungsten tip (anode) and stainless steel tube surrounding the tip (cathode), was employed. There was no detectable fluorescence in the area immediately surrounding stimulating electrodes during *in vitro* tests of dye solutions without slices. The stimulation intensity applied was between 100 and 300 μA, a range which yielded distinguishable responses over 1–2 s (100 Hz) with a minimum appearance of air bubbles.

The indicators used were Newport Green™ DCF (dipotassium salt), FluoZin-3 (tetrapotassium salt) (Invitrogen-Molecular probes, Eugene, Oregon), and Zinpyr-4 (Neurobiotex, Galveston, Texas). For confocal fluorescence microscopy and data acquisition, images were obtained utilizing an inverted Zeiss Axiovert LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany). The objective lens used was 10× with a 0.3 NA. Z-series scanning was performed in order to focus on middle layer of the tissue slice, thereby avoiding the error prone analysis of surface cells damaged during tissue processing. We determined the average value of basal fluorescence from individual regions of interest (ROIs) before electrical stimulation. The change in fluorescence intensity, ΔF (range = difference between the fluorescence at a given time of interest and the average basal fluorescence) was plotted to make the baselines comparable. Since each experiment lasted for only about 50–60 s (total fifty images), the effect of photobleaching was negligible. Statistical analysis was done using One-way ANOVA in cases where mentioned.

Although reliable responses to electrical stimulation could be recorded with the electrode in any portion of the CA3-hilus region of the hippocampus, the electrode was preferentially placed in the mossy fiber pathway in the hilar region closer to granule cells but still outside of the granule cell layer (Fig. 1A and B). This was the ideal location to compare the difference in fluorescence intensity between hilus-CA3 regions of the dentate gyrus and the molecular layer. The hippocampal mossy fibers make connections with neurons in the hilus and with pyramidal neurons in CA3 but do not innervate the molecular layer of

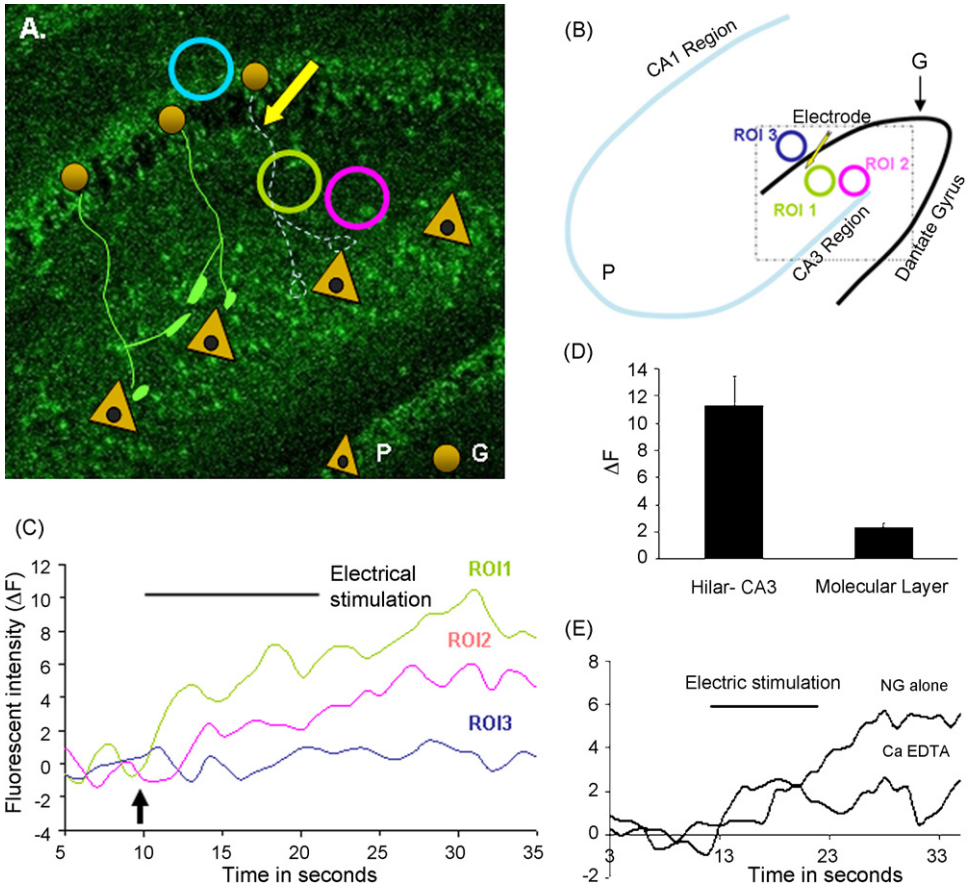

Fig. 1. Synaptically released Zn^{2+} induced by electrical stimulation at the mossy fiber pathway. (A) Images of the hilus of the hippocampal dentate gyrus perfused with 10 μM NG. The yellow arrow represents the tip of the electrode placed in the hilar region close to the granule cells. The three circles represent the three regions of interest (ROIs) studied. (B) Depiction of a hippocampal slice. Three open circles represent ROIs, the number of which corresponds with ROIs in A and with the curves plotted in C. (C) Electrical stimulation (100 Hz) evoked release of Zn^{2+} from mossy fiber terminals measured by changes in fluorescence intensity. Changes of fluorescence over time at three separate ROIs are plotted as curves ROI1, ROI2, and ROI3. Arrow indicates the beginning of stimulation. (D) Summary of NG fluorescence responses in hilus and molecular layer to the same source of electrical stimulation. Values plotted are the mean ± S.E.M., $N = 6$; $p < 0.01$. (E) The Zn^{2+} chelator CaEDTA (1 mM) inhibited the NG fluorescent response to electrical stimulation.

dentate gyrus. Vesicular Zn^{2+} is concentrated in the giant axonal boutons of mossy fibers. Therefore, we did not expect to detect increases in Zn^{2+}-specific fluorescence in the molecular layer.

Among the three Zn^{2+} indicators employed in the present study, NG is the commonly used and well-characterized selective fluorescent Zn^{2+} indicator. More importantly, NG does not appreciably fluoresce in the presence of high concentrations (in mM) of Ca^{2+} and Mg^{2+} [7,8]. Electrically stimulated slices showed reliable fluorescent responses in the CA3-hilar region. The Zn^{2+} chelator CaEDTA inhibited this fluorescent signal (Fig. 1E). In general, Zn^{2+} release induced by stimulation could be detected up to several hundred μm along the trajectory of the mossy fibers (from granule cells to CA3) with substantial fluorescent responses being acquired in the CA3 stratum lucidum where mossy fibers are compactly distributed.

Our results show a distinctive spatial pattern of NG fluorescence in response to electrical stimulation at the dentate gyrus of the hippocampus. As shown in Fig. 1A–C, of three adjacent ROIs, the greatest fluorescence intensities was always detected adjacent to the electrode (ROI1), with lower amplitudes of fluorescence increases observed at greater distances (ROI2). We did not observe any perceptible delay in the onset of fluorescence increases in the ROIs along mossy fiber projections, indicating the responsiveness of NG to fast neuronal activity evoked by electrical stimulation. Presumably, the smaller fluorescence intensity observed at distances far from the stimulating electrode is due to the cutting of axons during preparation of the slices. The fluorescence of the granule cell dendrites located in the molecular layer of the dentate gyrus (ROI3) did not increase after stimulation (Fig. 1D). Like ROI1, ROI3 was also adjacent to the electrodes but in a position outside the hilus. This spatial pattern of NG fluorescence is consistent with synaptically released Zn^{2+} from hippocampal mossy fiber terminals.

To further ensure that the NG responses observed were the result of Zn^{2+} release from Zn^{2+}-containing mossy fiber boutons, we examined the change of NG fluorescence in CA1 region of hippocampus by stimulation of Schaffer collaterals which form synapses with CA1 pyramidal neurons. We observed rising fluorescence only from regions close to the CA1 pyramidal neurons (the inner 1/3 of stratum radiatum). However, the fluorescence increase was less than 30% (2.43 ± 1.015, $p < 0.05$) of that recorded in CA3. These data indicate the existence of a low level of Zn^{2+}-containing terminals in the CA1 region [10].

Zinpyr-4, like NG, did not exhibit a substantial change in fluorescence to mM concentrations of Ca^{2+} or Mg^{2+} [8]. Although ostensibly appearing to be an excellent extracellular Zn^{2+} indicator, unlike NG, we found that Zinpyr-4 loaded hippocampal slices did not show any response to electrical stimulation (Fig. 2A). We attributed the difference in fluorescent responses from the two indicators to the difference in their sensitivities to Zn^{2+}. Zinpyr-4 (Zn^{2+} $K_D = 1$ nM) could be saturated at relatively lower concentrations (~1 μM) (Fig 2B without slice). We hypothesized that Zn^{2+} contamination in the brain slice preparations is a factor affecting extracellular Zn^{2+} detection, especially with high-affinity indicators like Zinpyr-4. This hypothesis was tested by adding exogenous Zn^{2+} directly onto Zinpyr-4 loaded hippocampal slices. Had Zinpyr-4 been able to

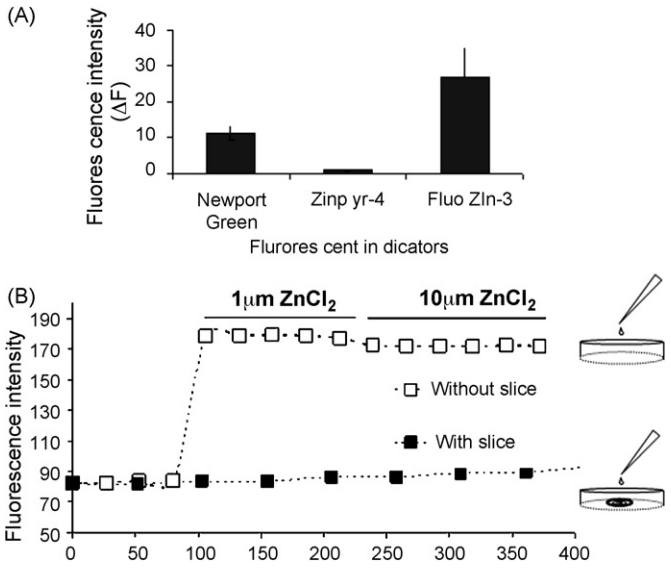

Fig. 2. Application of Zinpyr-4 in detecting synaptically released Zn^{2+} in response to electrical stimulation. (A) Increase in Newport Green, Zinpyr-4 and FluoZin-3 fluorescence evoked by electrical stimulation. Values plotted are the mean ± S.E.M., $N = 6$ (NG), 5 (Zinpyr-4), 6 (FluoZin-3); $p < 0.01$. (B) A representative experiment showing the effect of exogenous Zn^{2+} added into the recording chamber without and with a hippocampal slice. For *in vitro* tests, Zn^{2+} was added to ACSF that already contained 10 μM Zinpyr-4. Additions of 1 μM or 10 μM Zn^{2+} did not change fluorescence intensity of the hippocampal slice loaded with Zinpyr-4.

detect extracellular Zn^{2+}, we would have observed an increase in the fluorescence induced by exogenous Zn^{2+}. There was, however, little change in Zinpyr-4 fluorescence in the presence of either 1 μM or 10 μM Zn^{2+} (Fig. 2B). In contrast, NG loaded slices showed a stepwise increase in fluorescence after adding 1, 10, and 100 μM Zn^{2+} (data not shown, but see [7,8]), indicating that NG was not saturated with Zn^{2+} contamination.

FluoZin-3 was chosen in this study because it has a high-affinity to Zn^{2+} ($K_D = 15$ nM) and, unlike Zinpyr-4 and Newport Green, is also sensitive to Ca^{2+}. FluoZin-3 yields appreciable fluorescence after binding in 100 μM Ca^{2+} [4,8]. We initially noticed the Ca^{2+} sensitivity of FluoZin-3 when we observed bright green fluorescence in physiological medium (ACSF) containing 2.5 mM Ca^{2+}. Therefore, the background fluorescence of FluoZin-3 perfused hippocampal slices was higher than that of NG or Zinpyr-4 perfused slices. When applying electrical stimulation to the hippocampal slice, unlike the high-affinity indicator Zinpyr-4, we recorded a marked increase in FluoZin-3 florescence intensity (Figs. 2A and 3A). Comparison of the two indicators showed that FluoZin-3 yielded a higher increase as compared to NG. However, we did not record a distinctive spatial pattern of fluorescence responses as observed with NG; rather, there was a substantial increase in FluoZin-3 fluorescence in the molecular region of dentate gyrus as well.

Though hippocampal slices bathed in FluoZin-3 demonstrated large responses to electrical stimulation, the additions of 10 and 100 μM Zn^{2+} to 10 μM FluoZin-3 solution yielded no further increase in fluorescence intensity (Fig. 3B without slices). This was consistent with our previous result showing the response of FluoZin-3 to Zn^{2+} reaches a plateau with the

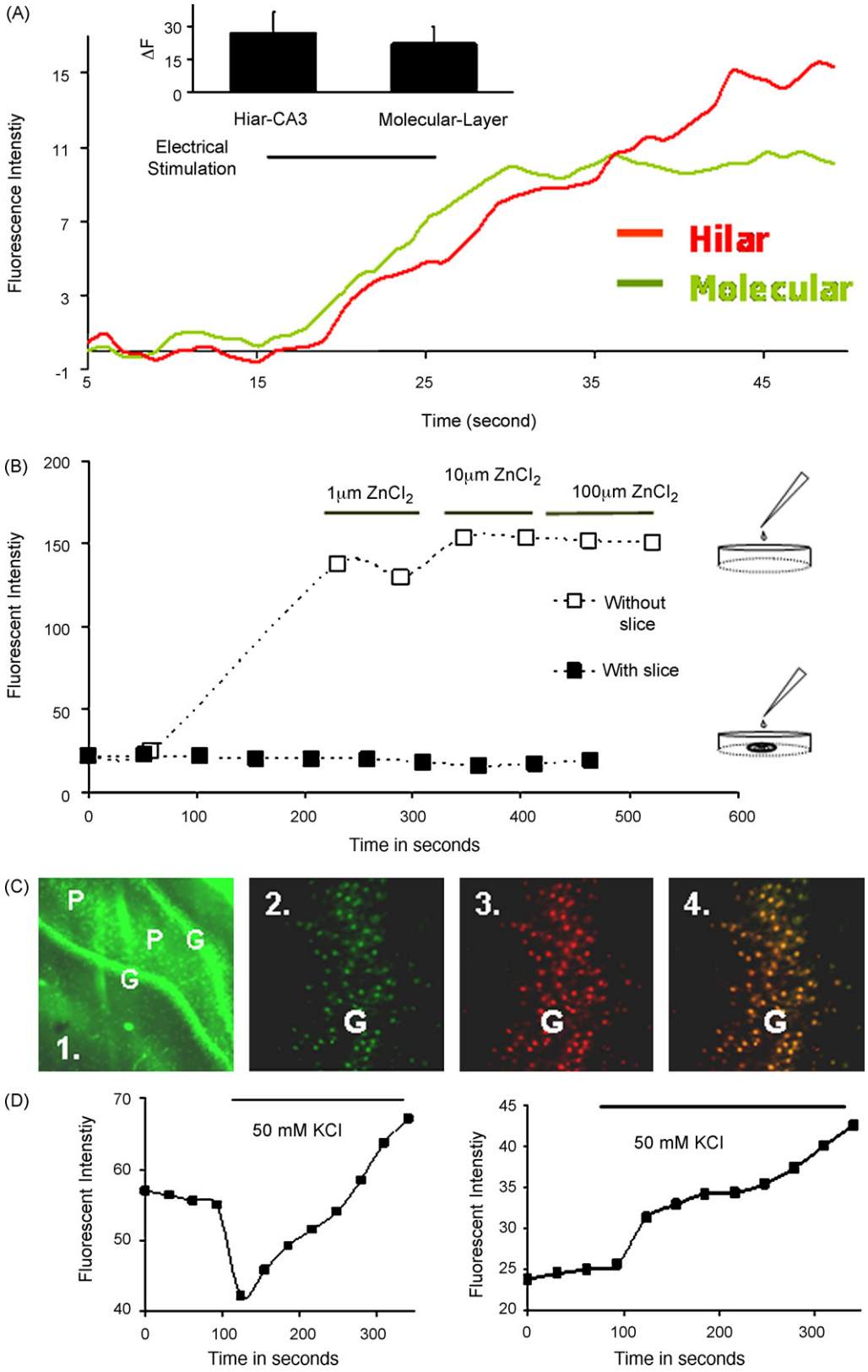

Fig. 3. Stimulation-induced increase of FluoZin-3 fluorescence. (A) Electrical stimulation (100 Hz for 10 s) in the mossy fiber pathways evoked increases in FluoZin-3 fluorescence. However, there was an increase in response in the hilar-CA3 region as well as the molecular region. The placement of the electrode was the same as in Fig. 1. *Insert*: The average value of fluorescence increases (ΔF) in hippocampal regions with high frequency electrical stimulation in the presence of FluoZin-3. (B) Representative experiments showing the fluorescence responses of FluoZin-3 to exogenous Zn^{2+}. In the absence of hippocampal slices, FluoZin-3 is saturated at about 1 μM zinc concentration. With the hippocampal slice present, addition of 1 μM or 10 μM or 100 μM did not change FluoZin-3 fluorescence. (C) Confocal microscopic fluorescence images of hippocampal sections stained with FluoZin-3 and PI. (1) An image of the dentate gyrus of a hippocampal slice loaded with

probe becoming saturated at about 1 μM of Zn^{2+} [8]. Similar to results observed with Zinpyr-4, there was little change of fluorescence when exogenous Zn^{2+} was directly applied to FluoZin-3 loaded hippocampal slices (Fig. 3B with slices). If FluoZin-3 was capable of detecting extracellular Zn^{2+}, we would have observed an increase in fluorescence induced by the addition of exogenous Zn^{2+}. Taken together, these results suggest that under our experimental conditions, the response of FluoZin-3 to electrical stimulation cannot be explained by synaptically released Zn^{2+} from hippocampal mossy fibers alone.

The loading FluoZin-3 yields a very bright background fluorescence that can observe in every tissue section with a conventional light fluorescent microscope (see also [6]). Using high-resolution confocal microscopy, we also observed the bright background fluorescence of FluoZin-3 (Fig. 3C), most notably in laminar structures formed by compact cell bodies such as the pyramidal neurons and granule cells of hippocampus. Co-localization studies with propidium iodide (PI), an accepted measure of apoptotic cell death, revealed that background fluorescence was due to FluoZin-3 staining damaged cells that had lost their membrane integrity. Investigation with other cell-impermeable Zn^{2+} indicators revealed similar patterns of background fluorescence [11].

To understand the possible effects of Ca^{2+} on extracellular FluoZin-3 fluorescence, we perfused hippocampal slices with 50 mM KCl to depolarize neuronal membrane. 50 μM of CaEDTA was also employed to minimize contaminant zinc. The hilar region was studied for changes in fluorescence. As seen in Fig. 3D, there was an initial decrease followed by a delayed increase in FluoZin-3 fluorescence. We are attributing this initial decrease to the sudden reduction of extracellular calcium due to the massive depolarization of all of the viable neurons in the slice (Ca^{2+} influx through voltage-dependent calcium channels). Moreover, these data suggested that at least a part of the FluoZin-3 fluorescence was due to calcium (2.5 mM) present in the medium.

The data in this study support the presence of synaptically released Zn^{2+} in the hippocampal mossy fiber-CA3 region. We found that extracellular Zn^{2+} released from the mossy fiber terminals after high frequency electrical stimulation can be detected by a relatively low-affinity indicator like NG. However, Zinpyr-4, a high-affinity fluorescent Zn^{2+} indicator, showed no response to electrical stimulation. Another high-affinity fluorescent Zn^{2+} indicator FluoZin-3 showed an increase in fluorescence intensity to electrical stimulation of the hippocampal mossy fiber pathways. The FluoZin-3 fluorescence response was larger than what we had observed with NG. Intriguingly, the increase in response to FluoZin-3 recorded in the molecular region of dentate gyrus where mossy fibers do not innervate. The addition of exogenous Zn^{2+} on Zinpyr-4 or FluoZin-3 loaded slices did not yield any increase in fluorescence. This was probably due to the saturation of Zinpyr-4 or FluoZin-3 with Zn^{2+} contaminants already present in hippocampal slices. These data suggest that the kinetics of a probe may affect individual outcomes of the Zn^{2+} detection in the extracellular space.

Detection of synaptically released Zn^{2+} with NG highlights a distinctive pattern of fluorescent responses in the dentate gyrus of hippocampal slices (Fig. 1). The lack of response in the molecular layer supports the theory that vesicular Zn^{2+} is released only along the Zn^{2+}-containing mossy fiber projection. This result could not have occurred if glial cells or dendrites had transmitted to the spread of Zn^{2+} or if contaminating ions had been released from the electrode during stimulation. Similarly, if fluorescence measurements recorded were the result of fluorescence intrinsic to the hippocampal slices (such as those produced from NADPH), we would have anticipated an evenly spread or circular NG fluorescence from the electrode. Therefore, the most rational explanation of NG fluorescence is that it detected a substance whose release was elicited by electrical stimulation and which followed the trajectories of mossy fibers in the hippocampus. The substance released is most likely Zn^{2+} due to (1) the selectivity of NG to Zn^{2+}, (2) the distinctive pattern of NG fluorescence from Zn^{2+}-containing mossy fibers, and (3) the sensitivity of NG fluorescence to Zn^{2+} chelator CaEDTA.

When selecting fluorescent probes, one tends to seek a high-affinity probe over a low-affinity probe. However, our study indicates that Zn^{2+} contamination in brain slice preparations is a factor affecting Zn^{2+} detection with fluorescence indicators, especially high-affinity indicators like Zinpyr-4 and FluoZin-3. High-affinity indicators are less problematic when detecting intracellular Zn^{2+} ($[Zn^{2+}]_i$) since $[Zn^{2+}]_i$ at resting levels is considered to be very low. However, these probes may be less effective when measuring extracellular or synaptically released Zn^{2+} in the extracellular space as contaminating levels of Zn^{2+} can saturate the probe, masking its ability to detect Zn^{2+} release during neuronal activities. Every probe can detect Zn^{2+} over a different range, but most are sensitive to changes in concentration within 1–2 orders of their respective K_D values. This notion is with our results. NG can detect Zn^{2+} without becoming saturated up to 100 μM. Zinpyr-4 and FluoZin-3 are saturated at relatively lower concentrations of Zn^{2+} (1 μM, [8]).

Zinc contamination can occur from items such as glassware, tubing, electrodes, or other lab-ware [5]; however, a significant amount of contamination originates from injured cells in hippocampal slice itself. In addition, hypoxic damage could trigger cellular death, leading to Zn^{2+} contamination during slicing and dye loading (this problem becoming significant with slices >200 μm). To overcome the dilemma of Zn^{2+} contamination, a Zn^{2+} chelator such as CaEDTA can be used in the perfusion medium to chelate Zn^{2+} residues [6,9]. One concern is that the chelator would compete with the fluorescent indicator for binding with Zn^{2+} (for example, CaEDTA versus FluoZin-

cell-impermeable FluoZin-3. (2) Image of the same section in (1) viewed at 25×, representing FluoZin-3 stained granule cell bodies green with emission at 505 nm. (3) PI stained red nuclei with emission at 645 nm. (4) Composite overlay fluorescence images showing co-localization of PI nuclei within cell bodies labeled with FluoZin-3. P, pyramidal cells; G granule cells. (D) Two representative tracings of the change in fluorescence observed in the hilar region when 50 mM KCl was added to the medium. The application of KCl gave an initial decrease in FluoZin-3 fluorescence (left) and persistent increase in NG fluorescence (right).

3, $K_{D\ CaEDTA} = 10^{-16.4}$ M versus $K_{D\ FluoZin\text{-}3} = 15^{-9}$ M). This competition could limit the capacity of an indicator to detect synaptically released Zn^{2+}. Another problem with this approach is that prolonged exposure to the chelator may eventually deplete vesicular Zn^{2+} as well [3].

Unlike the high-affinity indicator Zinpyr-4, FluoZin-3 showed a large increase in fluorescent response during electrical stimulation. The FluoZin-3 fluorescent response was larger than what we observed with NG. Incidentally, the fluorescence response of FluoZin-3 spread even in the molecular region of the dentate gyrus where there is no mossy fiber innervation. Given its fluorescence pattern, FluoZin-3 fluorescence seems not to be derived from its binding with Zn^{2+} in our experimental condition. This conclusion is further supported by the observation that addition of exogenous Zn^{2+} onto the FluoZin-3 loaded slice gave little change in fluorescence.

On the other hand, 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. FluoZin-3 showed a large response to Ca^{2+} which was about 30% of the maximum response of FluoZin-3 to Zn^{2+} [8] (but see also [1]). FluoZin-3, not other two indicators, gave a bright green fluorescence to the physiological medium (ACSF) bathing the slices, which contained 2.5 mM Ca^{2+}. According to the manufacture (Molecular Probes), where we obtained the probe, the Zn^{2+}-binding affinity of FluoZin-3 is unperturbed by Ca^{2+} concentrations up to at least 1 μM [4]. Thus, it is critical to be aware of the specificity or selectivity of a probe to a targeted ion (such as Zn^{2+}) in the presence of potential interferent (such as Ca^{2+}).

In summary, fluorescent extracellular Zn^{2+} imaging with FluoZin-3 can be somewhat unpredictable. Using FluoZin-3, Kay showed that little Zn^{2+} is released into the extracellular space during the course of synaptic transmission [6]. Also, using FluoZin-3 model, Qian and Noebels [9] have reported significant increases in extracellular Zn^{2+} and suggested that Zn^{2+} is co-released with glutamate during synaptic transmission. They used a low concentration of CaEDTA to circumvent the problem of saturation of the indicator. Another puzzle related to FluoZin-3 is its bright background fluorescence which has been described as the possible externalization of membrane bound Zn^{2+} to the extracellular space. Using confocal microscopy we reveal that this background fluorescence is due to FluoZin-3 staining damaged cells that have lost their membrane integrity (Fig. 3C, [11]). Our data suggest that the selectivity and sensitivity of a specific indicator affects the outcome of experimental results, and hence the interpretation. This conclusion highlights the need for careful evaluation of any probe prior to their use in a given experimental system.

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