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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

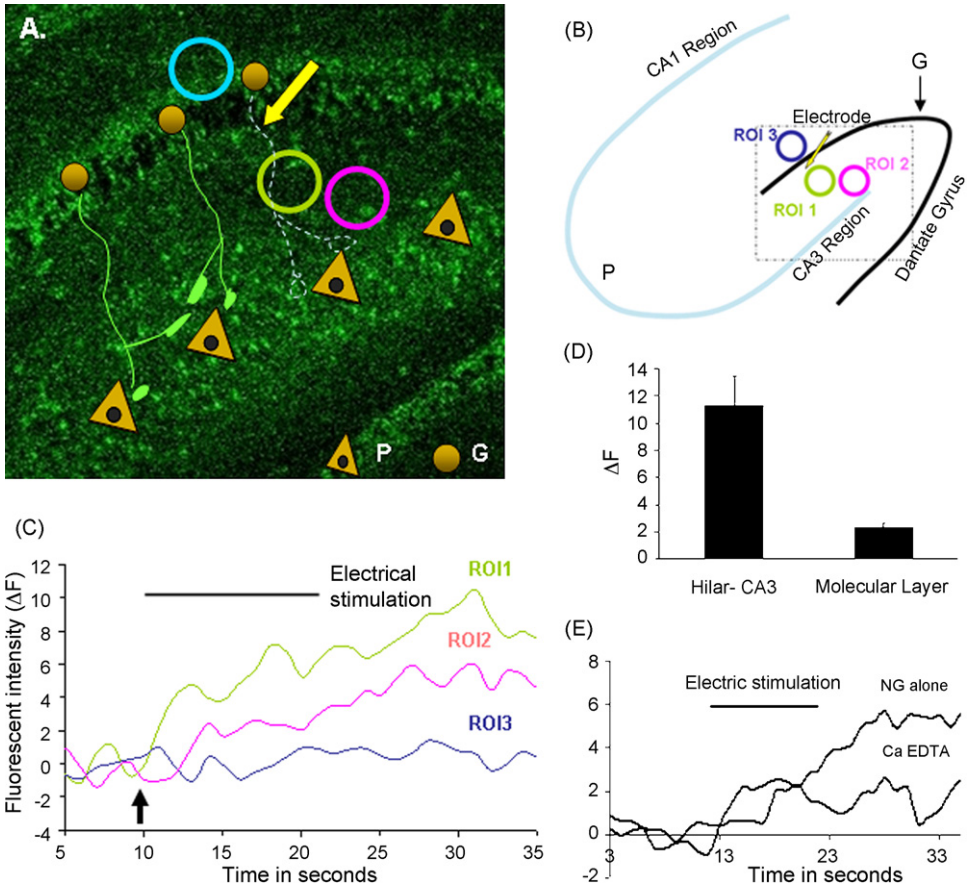

Fig. 1. Synaptically released Zn^{2+} induced by electrical stimulation at the mossy fiber pathway. (A) Images of the hilus of the hippocampal dentate gyrus perfused with 10 μM NG. The yellow arrow represents the tip of the electrode placed in the hilar region close to the granule cells. The three circles 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.