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Two-photon imaging of Zn\textsuperscript{2+} dynamics in mossy fiber boutons of adult hippocampal slices

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Mossy fiber termini in the hippocampus accumulate Zn\textsuperscript{2+}, which is released with glutamate from synaptic vesicles upon neural excitation. Understanding the spatiotemporal regulation of mobile Zn\textsuperscript{2+} at the synaptic level is challenging owing to the difficulty of visualizing Zn\textsuperscript{2+} at individual synapses. Here we describe the use of zinc-responsive fluorescent probes together with two-photon microscopy to image Zn\textsuperscript{2+} dynamics mediated by NMDA receptor-dependent long-term potentiation induction at single mossy fiber termini of dentate gyrus neurons in adult mouse hippocampal slices. The membrane-impermeable fluorescent Zn\textsuperscript{2+} probe, 6-CO\textsubscript{2}H-ZAP4, was loaded into presynaptic vesicles in hippocampal mossy fiber termini upon KCl-induced depolarization, which triggers subsequent endocytosis and vesicular restoration. Local tetanic stimulation decreased the Zn\textsuperscript{2+} signal observed at individual presynaptic sites, indicating release of the Zn\textsuperscript{2+} from vesicles in synaptic potentiation. This synapse-level two-photon Zn\textsuperscript{2+} imaging method enables monitoring of presynaptic Zn\textsuperscript{2+} dynamics for improving the understanding of physiological roles of mobile Zn\textsuperscript{2+} in regular and aberrant neurologic function.

Although most cellular Zn\textsuperscript{2+} is sequestered within proteins, stores of loosely bound Zn\textsuperscript{2+} are present in many kinds of cells. This mobile Zn\textsuperscript{2+} pool is believed to mediate cellular processes, including neurotransmission (1). Within the hippocampus, mossy fibers connecting the dentate gyrus (DG) and the CA3 regions contain Zn\textsuperscript{2+} in glutamatergic synaptic vesicles. The precise concentration of Zn\textsuperscript{2+} within the neuronal vesicles is unknown, with upper estimates ranging in the low millimolar range (2, 3). Upon stimulation, Zn\textsuperscript{2+} is believed to be coreleased with glutamate and to modulate glutamate-dependent long-term potentiation induction at single synapses. Here we present the synthesis of the probe ZinAlkylPyr-4 (ZAP4) and its 6-carboxylic acid derivative (6-CO\textsubscript{2}H-ZAP4). The molecular structure of ZAP4 was modeled after earlier ZAP probes (17), with pentafluorobenzyl groups chosen as the alkyl groups to reduce the basicity of the probe and minimize proton-induced enhancement of the emission. The 6-CO\textsubscript{2}H-ZAP4 probe was constructed as a membrane-impermeant Zn\textsuperscript{2+} probe, analogous to the previously reported 6-CO\textsubscript{2}H-ZP1 (20).

Previous attempts to monitor presynaptic Zn\textsuperscript{2+} dynamics in living brain tissues used membrane-permeant probes to detect intracellular Zn\textsuperscript{2+} from populations of presynaptic terminals at relatively low spatial resolution at the tissue level (21, 22). In addition, activity-dependent extracellular Zn\textsuperscript{2+} release has been monitored with membrane-impermeant probes (16, 23–30). These tissue level-averaged Zn\textsuperscript{2+} imaging methods have limited spatial and temporal resolution and fail to specify the precise location of the relevant synapses.

We describe intracellular Zn\textsuperscript{2+} imaging at the single-synapse level in mossy fiber termini of neurons in acute hippocampal slices from adult mice. The technique relies on the membrane-impermeant fluorescent Zn\textsuperscript{2+} probe 6-CO\textsubscript{2}H-ZAP4 and two-photon fluorescence microscopy.

Results

Synthesis and Characterization of ZAP4 and 6-CO\textsubscript{2}H-ZAP4. To image the Zn\textsuperscript{2+} within single presynaptic structures, we first synthesized ZAP4 and its membrane-impermeant derivative 6-CO\textsubscript{2}H-ZAP4 (Fig. L4 and SI Appendix, Fig. S1). ZAP4 and 6-CO\textsubscript{2}H-ZAP4 can be isolated in good yields through Mannich reactions with

zinc ion | metalloneurochemistry

Significance

Zinc is essential to various organs, including the liver and brain. In the brain, Zn\textsuperscript{2+} is present at high concentrations and selectively accumulates in the presynaptic vesicles of glutamatergic neurons. Understanding the roles of zinc in these glutamatergic vesicles has been hampered by the inability to visualize zinc accumulation and release at individual synapses. We report a fluorescent zinc-responsive probe and a technique to selectively image mobile zinc in presynaptic vesicles. Using two-photon laser microscopy, we visualized Zn\textsuperscript{2+} at the synaptic level in tissues from adult mouse brains and successfully monitored Zn\textsuperscript{2+} release from individual neurons. Our probe and methodology will allow further studies into how Zn\textsuperscript{2+} regulates synaptic plasticity and other neurologic functions.
fluorescein precursors that are analogous to the procedures used to prepare ZP1 and ZAP1–3 probes (14, 17). The presence of carboxylic acid on the bottom ring does not appear to affect the synthetic reactions, for the yields of the probes are both ~60%. The isolation requires no chromatography, and ZAP4 can be crystallized from MeOH and its X-ray structure has been determined, making it the fourth metal-free Zinpyr probe to be structurally characterized to date (31). As with the previously crystallized probes and the Zn₂-ZP1 complex, the bottom carboxylate is locked into the lactone isomer (11, 31). Whether this isomer is the predominant species in solution or whether the lactone formation is a prerequisite for crystal growth is unclear.

**Photophysical Characterization of ZAP4 and 6-CO₂H-ZAP4.** The maximum absorption and emission wavelengths of metal-free ZAP4 are 514 nm (ε = 4,650 M⁻¹ cm⁻¹; SI Appendix, Fig. S2) and 527 nm (Φ = 0.19; Fig. 1B), respectively. The low absorptivity compared with other Zn²⁺ probes, such as Zinpyr-1 (ZP1 = 79,500 M⁻¹ cm⁻¹) (11), most likely results from aggregation of the probe. A similarly low absorptivity was observed for ZAP3, a previously reported ZAP probe with phenyl groups in place of the pentafluorophenyl groups (17). On dilution, the ε of the λ_max of ZAP3 increased to a value that was more typical of a ZP₂-type molecule, an observation consistent with the chromophores clustering at high concentrations. We did not observe the same phenomenon for ZAP4, even with the much lower concentrations that could be visualized with a 10-cm path length cuvette.

Upon addition of a large excess of Zn²⁺ (>1 mM ZnCl₂), the emission of ZAP4 increased by more than a factor of six (Fig. 1B). Concurrently, the absorption and emission wavelengths blue-shifted slightly to 502 nm (ε = 8,600 M⁻¹ cm⁻¹; SI Appendix, Fig. S2) and 520 nm (Φ = 0.47; Fig. 1B), respectively. After titration by optical spectroscopy, we could distinguish three relevant species: ZAP4, Zn₁A-ZAP4, and Zn₂B-ZAP4 (SI Appendix, Figs. S3 and S4). The metal-ligand stoichiometry of Zn₁A-ZAP4 and Zn₂B-ZAP4 cannot be assigned with certainty, because these species form only when a large excess of Zn²⁺ is present. Owing to its strong resemblance to ZP1 and other ditopic Zn²⁺ responsive probes, we speculate that the Zn₁A-ZAP4 species is most likely a 2:1 Zn²⁺-ligand complex, the formation of which is preceded by that of a 1:1 complex that is spectroscopically similar to metal-free ZAP4 (31, 32). The absorption intensity of the Zn₁A-ZAP4 species was greater than that of either ZAP4 or Zn₂B-ZAP4; it is possible that additional equivalents of Zn²⁺ increase absorption by triggering the dissociation of ZAP4 aggregates. Titrations with ZnCl₂ revealed two plateaus in the emission response, one at 30–60 μM and the other at >2 mM zinc (Fig. 1B). Of the divalent metal ions tested, only Zn²⁺ and Cd²⁺ induced a fluorometric enhancement in a 1.0 μM solution of ZAP4. The presence of 100 μM Mg²⁺, Ca²⁺, or Mn²⁺ did not significantly alter the fluorometric response to Zn²⁺ (Fig. 1E).

The 6-CO₂H-ZAP4 probe had absorption maxima at 516 nm (ε = 60,600 M⁻¹ cm⁻¹) and 336 nm (Φ = 7.050 M⁻¹ cm⁻¹) in 100 mM KCl and 50 mM Pipes (pH 7.0). The presence of the carboxylic acid on the bottom ring increased the probe absorptivity significantly.
possibly by disrupting sensor aggregation (17). The 6-CO₂H-H-ZAP4 excitation maximum of 516 nm is similar to that of the 515-nm band observed for ZP1 (79,500 M⁻¹ cm⁻¹) (11). Upon addition of a large excess (>1 mM) of ZnCl₂, the fluorescence of 6-CO₂H-H-ZAP4 increased by more than fivefold (Fig. 1C). The 516-nm absorption band shifted to 504 nm upon the addition of ZnCl₂, with only a slight decrease in intensity. The quantum yield increased from 0.14 to 0.62 upon saturation with Zn²⁺. The emission peak shifted from 527 nm to 523 nm (Fig. 1C). The spectroscopic changes can be plausibly assigned to the formation of a 2:1 Zn²⁺-6-CO₂H-H-ZAP4 complex.

**Determination of Probe Affinities for Zn²⁺.** Deconvolution of the changes occurring in both the optical and fluorescence spectra of ZAP4 upon Zn²⁺ chelation yielded dissociation constants (Kd) of 23 ± 7 μM for ZnA⁻ZAP4 and 0.54 ± 0.15 mM for ZnB⁻ZAP4 (SI Appendix, Fig. S2). The overlap of the two bending events precludes more accurate measurement of the Kd value associated with the ZnA⁻ZAP4 complex. Stopped-flow kinetic analysis of the first visualizable metal binding event finds a linear correlation between the observed rate constant (kobs) and the concentration of Zn²⁺, with a kₐ = 3.07 ± 0.18 × 10⁸ M⁻¹ s⁻¹ at 4.3 °C (SI Appendix, Fig. S7). The rate constant associated with the Zn²⁺ dissociation from the 10 species was 6.6 ± 2.0 s⁻¹. The Kd value of 22 ± 7 μM calculated from the two rate constants closely agrees with that obtained from the ZnCl₂ titrations at 25 °C. The 6-CO₂H derivative 6-CO₂H-H-ZAP4 differs substantially from ZAP4, exhibiting only a single photophysical response when monitored fluorimetrically. The associated Kd value of 22 ± 4 μM is equal within the limits of error to that found for the formation of ZnA⁻ZAP4 from ZAP4 (SI Appendix, Fig. S4).

**Impact of pH on Probe Emission.** As with previous probes with similar structures (11, 17), the emission of ZAP4 and 6-CO₂H-H-ZAP4 increased as the pH decreased from 12 to 5 (Fig. 1D). The fluorescence then decreased as the pH dropped from 5 to 2, a phenomenon associated with protonation of the benzoate ring carboxylate functionalities. The pH dependence of the emission was fit to a two-event model. The kₐ value associated with the increase in fluorescence was 7.0 ± 0.10 for ZAP4 and 7.12 ± 0.10 for 6-CO₂H-H-ZAP4, both more than 1 unit less than the 8.4 value for ZP1 (11) and almost 1 unit less than the 7.88 for nonfluorophore ZAP3 (17). The kₐ values associated with the acid-induced turnoff were 2.42 ± 0.10 for ZAP4 and 3.77 ± 0.10 for 6-CO₂H-H-ZAP4.

**Two-Photon Properties of 6-CO₂H-H-ZAP4 for Vesicular Zn²⁺ Imaging in Hippocampal Slices.** We evaluated the ability of 6-CO₂H-H-ZAP4 to image Zn²⁺ in living brain tissues and compared it with the membrane-permeant ZP1, which was used previously to study vesicular Zn²⁺ in olfactory bulb tissues (21). We first examined the excitation profiles using two-photon microscopy, which allows for fluorescence imaging of thicker living tissues (33). ZP1 solutions displayed two relatively weak excitation peaks at <700 nm and >1,000 nm in the absence of Zn²⁺ (Fig. 2A, no Zn²⁺). After the addition of 100 μM ZnCl₂, the fluorescence intensity increased substantially for all excitation wavelengths from 700 to 1,000 nm (Fig. 2A, 100 μM ZnCl₂). The 6-CO₂H-H-ZAP4 probe had a similar two-photon excitation profile in the absence of Zn²⁺ and displayed enhanced fluorescence emission after the addition of 100 μM ZnCl₂ (Fig. 2B).

To test the ability of these Zn²⁺ probes to function as sensors in brain tissue, we incubated acute hippocampal slices from the brains of adult mice with ZP1 (25 μM) or 6-CO₂H-H-ZAP4 (5 μM), washed out the excess probe, and then fluorometrically imaged regions remote from the mossy fibers in the slices (Fig. 2C). The tissue background two-photon excitation profiles for emission after application of both ZP1 and 6-CO₂H-H-ZAP4 were similar and minimal at 850–900 nm; thus, we selected 860 nm as the excitation wavelength for imaging Zn²⁺ in brain slices.

**Visualization of Presynaptic Zn²⁺ at the Hippocampal Mossy Fiber Termi-**

n of Adult Acute Slices. To image endogenous, presynaptic Zn²⁺ in mossy fiber termini, we incubated acutely prepared hippocampal slices with membrane-permeant ZP1. Intense fluorescent staining revealed the hilus of the dentate gyrus (DG) and the CA3 stratum lucidum in the mossy fiber region (Fig. 2D, Upper).
The membrane-impermeant probe 6-CO$_2$H-ZAP4 was loaded into bouton presynaptic vesicles through a protocol similar to that used for the lipophilic membrane probe FM1-43 (34, 35). Slices were immersed in artificial cerebrospinal fluid (ACSF) containing 5 μM 6-CO$_2$H-ZAP4 and 45 mM KCl for 15 min, which induces fusion of synaptic vesicles with the presynaptic membrane that are subsequently recycled and replenished with Zn$^{2+}$ and other vesicular content. The 6-CO$_2$H-ZAP4 is endocytosed during this process, becoming trapped within the newly formed synaptic vesicles because of its membrane impermeability. Excess 6-CO$_2$H-ZAP4 was removed by continuous perfusion with ACSF for at least 10 min. Two-photon fluorescence imaging of the slice revealed high accumulation of 6-CO$_2$H-ZAP4 in the stratum lucidum layer between the CA3 and DG relative to other hippocampal regions (Fig. 2D, Lower). The discontinuous mossy fiber staining is more evident in the higher-magnification images containing discrete puncta that most likely represent active boutons with Zn$^{2+}$ accumulation in their synaptic vesicles (Fig. 2E, Top).

To confirm that the puncta report Zn$^{2+}$, we applied the probes to brain slices obtained from zinc transporter 3 (ZnT3) knockout mice (36), which lack vesicular synaptic zinc (Fig. 2E, Middle and Bottom). Both ZP1 and 6-CO$_2$H-ZAP4 produced fluorescent puncta in the mossy fiber regions of ZnT3$^{+/+}$ and ZnT3$^{-/-}$ hippocampal slices, whereas staining was reduced in ZnT3$^{-/-}$ hippocampi to a similar intensity as in other hippocampal subregions.

We also examined the distribution of 6-CO$_2$H-ZAP4 fluorescent puncta in Thy1-Brainbow1.0 (line H) mice (37) that express a red fluorescent protein (tdimer2) in mossy fiber boutons. In slices from the Brainbow mice, neurons exhibiting sparse red fluorescence were detected in DG granule neurons and their mossy fiber axons (Fig. 2F). After treatment with 6-CO$_2$H-ZAP4, we detected some of the 6-CO$_2$H-ZAP4 fluorescent puncta (green) overlapped with red spots in the mossy fiber region (Fig. 2G, Upper); higher-magnification 3D images revealed colocalized puncta of 6-CO$_2$H-ZAP4 and tdimer2 (Fig. 2G, Lower). To confirm whether the 6-CO$_2$H-ZAP4 puncta are localized in presynaptic regions, we immunostained hippocampal slices with the presynaptic active zone marker bassoon (38) after staining with 6-CO$_2$H-ZAP4 (Fig. 2H). The 6-CO$_2$H-ZAP4 puncta were preserved even after fixation and permeabilization. The puncta overlapped with bright bassoon spots, indicating presynaptic localization of the 6-CO$_2$H-ZAP4 probe.

The addition of the membrane-permeant Zn$^{2+}$ chelator tris-(2-pyridylmethyl)amine (TPA) (21) reduced the fluorescence intensity of both ZP1 and 6-CO$_2$H-ZAP4 puncta compared with control slices continuously perfused with ACSF alone, indicating that both probes report Zn$^{2+}$ within boutons that contain synaptic vesicles (Fig. 3 A and B). These results demonstrate that both membrane-permeant ZP1 and membrane-impermeant 6-CO$_2$H-ZAP4 detect accumulated Zn$^{2+}$ within the vesicles of mossy fiber boutons from acutely prepared hippocampal slices of adult mice.

**Visualization of Activity-Dependent Release of Zn$^{2+}$ at the Mossy Fiber Termini of Adult Acute Slices.** To monitor activity-dependent Zn$^{2+}$ release in mossy fiber boutons, we first loaded synaptic vesicles with the zinc probes. The fluorescence intensity of ZP1-stained boutons dropped (by 11% per min) during continuous perfusion of ACSF (Fig. 3C), but was unaffected by a K$^+$-evoked depolarizing stimulus (ACSF containing 90 mM KCl for 90 s). (Scale bar: 5 μm.) (Right) Time course of the Zn$^{2+}$ fluorescence intensity change in mossy fiber bouton termini, normalized to the average intensity before the K$^+$-evoked depolarizing stimulus (magenta bar). DCG-IV (5 μM) was added in ACSF after 6-CO$_2$H-ZAP4 staining (Stim. + DCG-IV). ZP1 (n = 18), 6-CO$_2$H-ZAP4 (Stim.; n = 8), 6-CO$_2$H-ZAP4 + DCG-IV (Stim. + DCG-IV; n = 14), (E) Schematic drawing of the configuration for local tetanic electrical stimulation using a glass electrode in the slice. (F) Fluorescence image from a mossy fiber region of a living hippocampal slice stained with 6-CO$_2$H-ZAP4. The arrow represents the position of the inserted electrode tip. The mossy fiber regions in white squares near the electrode (Stim.) and control regions at a distance from the electrode (Contr.) are enlarged in G. (Scale bar: 5 μm.) (G) 6-CO$_2$H-ZAP4 fluorescence at two mossy fiber regions obtained before and immediately after tetanic stimulation. (Scale bar: 5 μm.) (H) Time course of the fluorescence intensity change averaged from boutons and normalized to prestimulation levels. Control (Contr.; black circles; n = 8); mossy fiber region >30 μm away from stimulation points (glass electrode). Stimulation (Stim.; magenta circles; n = 8); mossy fiber region 5–15 μm (stimulation range) away from the electrode.
from a cluster of labeled boutons. On local tetanic electrical stimulation (42, 44) (Fig. 3E), 6-CO$_2$H-ZAP4 emissions were decreased at regions near the electrode (stimulated), but not at distant sites (control) within the same slices (Fig. 3 F–H). These results reveal that 6-CO$_2$H-ZAP4 monitors activity-dependent Zn$^{2+}$ release from mossy fiber boutons.

Discussion

Development of Zn$^{2+}$ Probes. The ZAP4 and 6-CO$_2$H-ZAP4 probes were designed to be less-basic analogs of previously reported ZAP sensors (17). The higher basicity of those sensors precluded them from detecting Zn$^{2+}$ at neutral pH. The target compounds were synthesized through well-established steps and can be isolated in good yields without chromatography.

The ZAP4 sensor has a notably low molar extinction coefficient for the main feature at 514 nm ($\varepsilon = 4,650$ M$^{-1}$ cm$^{-1}$). The next-weakest major band among Zinpyr-type fluorescein probes is that of ZAP3 (510 nm; $\varepsilon = 23,700$ M$^{-1}$ cm$^{-1}$) (17). Derivatives of fluorescein that enforce lactone formation are commonly colorless. The ability to crystallize this form of the metal-free probe may indicate a larger portion of this isomer in solution, which would explain its weak absorbance. Alternatively, aggregation of the sensor in solution may affect absorbance; a similar process is suspected to occur for ZAP3 (17), which exhibits a higher $\varepsilon$ value at lower metal ion concentration. Curiously, 6-CO$_2$H-ZAP4 has a much stronger band at 516 nm ($\varepsilon = 60,600$ M$^{-1}$ cm$^{-1}$) compared with ZAP4; otherwise, the photophysical features, particularly the quantum yields of the metal-free and Zn$^{2+}$-saturated forms, are similar.

The similarity extends to the basics of the metal-free probes and the dissociation constants associated with the first observable Zn$^{2+}$-binding event, and the carbocyclic acid installed on the bottom ring of 6-CO$_2$H-ZAP4 does not appear to affect the cation-binding properties of the two metal-binding domains. The pK$_a$ values for the proton-induced turn-on for ZAP4 and 6-CO$_2$H-ZAP4 are within the limits of error of each other. The pentabenzyl substituents successfully decrease these values relative to those reported for ZAP1-3, and enable fluorometric Zn$^{2+}$ detection at pH 7.0 (22). The binding affinities associated with the first observable Zn$^{2+}$-binding event are also identical within the limits of error of each other. The fluorescence responses likely correspond to the formation of 2:1 Zn$^{2+}$-ligand complexes, given that metallation of both metal-binding domains was required for the emissive response in previously described ditopic Zn$^{2+}$ sensors (31, 32). In these systems, the 1:1 Zn$^{2+}$-ligand complex was too spectroscopically similar to the metal-free probe to allow observation by a simple fluorimetric titration. The pK$_d$ values for the 2:1 Zn$^{2+}$-ligand complexes of ZAP4 (23 $\mu$M) and 6-CO$_2$H-ZAP4 (22 $\mu$M) are similar in magnitude to those measured for the OZ family (18).

Stopped-flow kinetic studies on the first observable association reaction between ZAP4 and Zn$^{2+}$ suggest a first-order dependence on the metal ion, and the reaction is most simply interpreted as a bimolecular collision. The equilibrium constant measured from the forward and backward rates agrees well with that obtained from singular value decomposition analysis of the titrations. The rate constant associated with the dissociation is greater than those measured for ZP1 probes, but less than those measured for OZ probes, as is the rate of Zn$^{2+}$ release (18).

The rapid metal ion release renders the probe less susceptible to metal contamination, much like the OZ and Zinspy probes (18, 19) and, to a lesser extent, the Me$_2$ZP1 probe (15). The ZAP4 sensor is capable of detecting Zn$^{2+}$ in the presence of an equimolar concentration of Fe$^{2+}$. Equimolar concentrations of Co$^{2+}$, Ni$^{2+}$, and Cu$^{2+}$ prevent the emission from exceeding that of the metal-free probe. The very low cellular concentrations of these free ions should not affect biological imaging with ZAP4 and 6-CO$_2$H-ZAP4.

Based on their pK$_d$ values and significant fluorescent responses to Zn$^{2+}$, these probes have a potential use in quantifying cellular Zn$^{2+}$ concentrations. However, the membrane-permeant ZAP4 has a sluggish second binding event, which can complicate interpretation of the fluorescence response. Moreover, the much weaker absorption of ZAP4 severely limits the brightness of its emission and thus its applicability to the study of biological processes. Conversely, the membrane-impermeant 6-CO$_2$H-ZAP4 has a single photophysical response for Zn$^{2+}$ and a much stronger absorption maximum than ZAP4.

Application of Zn$^{2+}$ Probes in Adult Mouse Acute Hippocampal Slices. The ability of 6-CO$_2$H-ZAP4 to successfully image synaptic vesicle Zn$^{2+}$ in the mossy fiber boutons of hippocampal neurons with two-photon fluorescence microscopy has several important implications. Two-photon fluorescence microscopy enables deep-tissue Zn$^{2+}$ imaging and avoids imaging non-specific fluorescence on the slice surface. The Zn$^{2+}$ images displayed here are of sufficiently high resolution for tracking individual synaptic termini. Because 6-CO$_2$H-ZAP4 is membrane-impermeant, its introduction into synaptic vesicles requires neuronal depolarization, with the probe entering the vesicles during the subsequent recycling event (Fig. 4A). The resultant bright fluorescence signal may be somewhat enhanced by proton-induced probe turn-on within the acidic vesicles (pH 5.0), the contribution of which can be estimated by the results from the described Zn$^{2+}$ T3 knockout mouse study.

Fig. 4. Models for presynaptic Zn$^{2+}$ imaging method at mossy fiber termini using Zn$^{2+}$ probes. (A, Left) Schematic model of presynaptic vesicle Zn$^{2+}$ staining using a membrane-impermeant Zn$^{2+}$ probe. Weak depolarization stimulus induces fusion of some synaptic vesicles that allows membrane-impermeant 6-CO$_2$H-ZAP4 to enter into the fused vesicles. The fluorescence probe then binds to Zn$^{2+}$ concentrated within vesicles by Zn$^{2+}$ transporters localized to the vesicle membrane. (Right) Schematic model of Zn$^{2+}$ release in mossy fiber termini during electric stimulation. Tetanic stimulation induces collapse fusion events of synaptic vesicles into the bouton membrane which is sufficient to release a large proportion of Zn$^{2+}$ (along with the fluorescent probe) from synaptic vesicles. (B) Model for Zn$^{2+}$ staining with the cell membrane-permeant fluorescence probe in mossy fiber termini. (Left) The Zn$^{2+}$ probe (ZP1) stains Zn$^{2+}$ within vesicles without synaptic activation owing to its membrane permeability. (Right) The membrane-permeant probe ZP1 stains Zn$^{2+}$-containing vesicles throughout the mossy fiber bouton. The mossy fiber bouton contains multiple active zones (cyan region).
The mossy fiber bouton is quite large (2–5 μm diameter) and contains ~16,000 synaptic vesicles (43). Synaptic activation most likely releases only a limited number of these vesicles, perhaps mainly near active zones. The ZP1 probe is membrane-permeant and able to stain all Zn$^{2+}$-containing vesicles (Fig. 4B). This property effectively reduces its sensitivity in detecting Zn$^{2+}$ dynamics and limits its use solely to the detection of the massive Zn$^{2+}$ release expected during strong electric stimulation in tissue-level assays (21). However, for synapse-level Zn$^{2+}$ imaging, local electric stimulation (42, 44) and highly magnified observation optics are needed to measure Zn$^{2+}$. Because 6-CO$_2$H-ZAP4 stains Zn$^{2+}$ only in actively recycled vesicles, it may be retained in vesicles near active zones, which are more likely to be released on stimulation. This phenomenon may allow 6-CO$_2$H-ZAP4 to more efficiently detect release of Zn$^{2+}$ relative to ZP1 and is one likely reason for the discrepancy in the ability of ZP1 and 6-CO$_2$H-ZAP4 to monitor K$^+$-evoked Zn$^{2+}$ release. Thus, 6-CO$_2$H-ZAP4, in combination with two-photon imaging, provides a unique tool for monitoring Zn$^{2+}$ release and dynamics in living tissues at single-synapse resolution. Our method will enable studies of presynaptic Zn$^{2+}$ function in synaptic plasticity, toxicity, and zincopathic brain disease.

Materials and Methods

Materials and procedures for all experiments are described in SI Appendix. Included in this appendix are the synthesis and characterization of compounds, neuronal imaging protocols, and two-photon microscopy experiments. Also provided are additional figures and tables relating to the characterization of compounds.

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