The influence of location of a fluorescent zinc-probe in brain slices on its response to synaptic activation

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Abstract

The precise role of the high concentration of ionic zinc found in the synaptic vesicles of certain glutamatergic terminals is unknown. Fluorescent probes with their ability to detect ions at low concentrations provide a powerful approach to monitoring cellular Zn²⁺ levels. In the last few years a number of fluorescent probes (indicators) have been synthesized that can be used to visualize Zn²⁺ in live cells. The interpretation of data gathered using such probes depends crucially on the location of the probe. Using acutely prepared hippocampal slices we provide evidence that the Zn²⁺-probes, ZnAF-2 and ZP4 are membrane permeant and are able to pass into synaptic vesicles. In addition, we show that changes in fluorescence of the Zn²⁺-probes can be used to monitor presynaptic activity, however, these changes are inconsistent with Zn²⁺ release.

Introduction

Ionic zinc plays diverse roles in cells, acting variously as enzymatic cofactor, structural element and second messenger (Smart et al. 2004). Because of its involvement in so many processes there is much interest in understanding the factors involved in governing its movement between cellular compartments (Finney and O'Halloran 2003). At present Zn²⁺-sensitive fluorimetric probes are the only tools available for visualizing the traffic of the ion within and between cells in live tissue (Jiang and Guo 2004; Kikuchi et al. 2004).

The synaptic terminations of the mossy fiber terminals, in mature animals, contain high concentrations of Zn²⁺ in synaptic vesicles (Frederickson 1989). The Zn²⁺ in these vesicles appears to be rather loosely bound, distinguishing it from the remaining ~90% of Zn²⁺ in neurons that is tightly associated with proteins. If a histochemical or fluorimetric probe is used to reveal the Zn²⁺ in slices, the terminal fields that are stained are...
distinguished by the characteristic ‘arrow-head’ of the hilus and the ‘hook’ of the mossy fibers. It has been proposed that synaptic Zn$^{2+}$ is released during the course of synaptic transmission and acts as a neuromodulator.

If Zn$^{2+}$ is released during the course of a synaptic event, a fluorescent Zn$^{2+}$ sensor in the extracellular space should respond with an increase in fluorescence. However, in order to establish that Zn$^{2+}$ is indeed released the location of the fluorescent probe has to be clearly proven and the chemical state of Zn$^{2+}$ within the terminals should, as we will show below, be taken into account.

As with human reporters, biological ones are dependent upon their location for constructing a ‘view’ on a biological event. Biological probes signal the experimentalist through changes in the intensity of fluorescence, or sometimes wavelength. Precisely what these changes mean depends on the nature of the probe and its locations within the tissue. If, for example, it is assumed that the probe is extracellular but is actually located in the cytosol, incorrect conclusions are likely to be drawn from the experiment.

How does one determine the location of a fluorescent probe? If a probe is located in the cytoplasm it is fairly easy to determine that this is so because the probe is simply evident as filling the cell body. However in deciding whether a probe is in the extracellular space or in intracellular vesicles there may be some ambiguity in identifying the location, if it is not possible to clearly resolve the cellular elements. Moreover, Zn$^{2+}$ may also be adsorbed to the outside of cells, forming what has been termed a Zn$^{2+}$-veneer (Kay 2003).

In order to determine the location of a probe, two types of chelators are required; one membrane permeant and the other impermeant. Whether the chelators are able to abstract Zn$^{2+}$ from the probe depends on the location of the probe and the relative affinity of the chelator. A chelator that is not membrane permeant and has a high affinity for Zn$^{2+}$ but does not change the physiological levels of calcium and magnesium, is Ca-EDTA, another is the recently introduced chelator EDPA (Kay 2003). A number of membrane permeant chelators are available, the most widely used being TPEN and DEDTC (diethyldithiocarbamate) (Danscher et al. 1975).

The di-(2-picolyl)amine (DPA) moiety has a high affinity for transition metals but little for calcium and magnesium, which has been harnessed in the widely used chelator TPEN (Arslan et al. 1985) and a number of fluorimetric probes (Jiang and Guo 2004). In this communication we determine the cellular location of two recently developed fluorimetric probes, ZnAF-2 (K$_d$ 2.7nM)(Hirano et al. 2000) and ZP4 (or Zinpyr-4, K$_d$ 0.65nM) (Burdette et al. 2003) in hippocampal slices. Both are monocarboxylated, employ DPA as the Zn$^{2+}$ chelator and have been reported to be membrane impermeant (Hirano et al. 2000) (Burdette et al. 2003).

**Materials and Methods**

**Hippocampal Slices**

Sprague-Dawley rat (16-60 day, male and female) were decapitated, the brain removed and placed in ice cold physiological saline containing (in mM); 125 NaCl, 2.5 KCl, 2 CaCl$_2$, 1.3 MgSO$_4$, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, and 25 glucose, bubbled with 95% O$_2$-5% CO$_2$. To combat Zn$^{2+}$ contamination high purity reagents were used, while avoiding metals, glass and plastics that can leach Zn$^{2+}$ into solutions (Kay 2004). Slices were cut at a thickness of 400 μm on a McIlwain tissue chopper, and held in an interface chamber at
room temperature for at least one hour prior to loading with the Zn$^{2+}$-probe. Slices were incubated in the interface chamber with the Zn$^{2+}$-probe from 0.5 to 3 hrs.

**Seizure induction**

Animals were injected intraperitoneally with 10 mg/kg kainic acid (Ocean Produce, Canada) dissolved in 0.9% NaCl. Control animals received a single injection of vehicle solution. 18 hours after the kainic acid injection rats were anesthetized with isoflurane and decapitated.

**Imaging live hippocampal slices**

The slices were stabilized with a U-shaped stainless steel piece cross strung with nylon fibers in a temperature controlled chamber (RC-27L; Warner Instruments). Images were acquired on an Olympus Optical BX50WI upright microscope. Illumination was provided by a monochromator set at 480 nm (T.I.L.L. Photonics), passed through a dichroic (Q495lp; Chroma Technology) and then through a filter (HQ530/60; Chroma Technology) onto the faceplate of a Princeton Instruments cooled CCD camera. Data were acquired by the MetaFluor program (Universal Imaging Corporation) and the images were analyzed using the ImageJ (NIH) program. No black-level adjustment was applied to images. A Picospritzer II (General Valves) was used to eject solution into slices.

Slices were loaded in an interface chamber by immersion in saline containing ZnAF-2 or ZP4. Fluorescent imaging experiments were performed in 1.5 ml of saline that was added to the chamber, stirred continuously with a jet of 95% O$_2$-5% CO$_2$ and held at 32 °C.

**Cryosections**

Brains were harvested and the two hippocampi frozen in dry ice and 2-methylbutane, then stored at –70 °C. 30 µm thick coronal sections were prepared using a cryostat (Lipshaw 1500) and mounted on prechilled glass slides coated with poly-L-lysine. The slices were incubated with 5 µM ZP4 in ACSF (concentrations in mM: NaCl 130, KCl 3.5, NaH$_2$PO$_4$ 1.25, MgSO$_4$ 5, CaCl$_2$ 1, NaHCO$_3$ 24 and glucose 10) for 90s. The slices were viewed on an Olympus BX51 microscope (excitation 470-480 nm, dichromatic beamsplitter 500 nm, bandpass filter: 515-550nm) and photographed with SPOT RT digital camera (Diagnostic Instruments Inc.). Images were analyzed with ImagePro software. The intensity of staining was quantified by \%$\Delta$F/Fo = \%($F-F_o$)/Fo; where F is the fluorescence intensity of a region with high vesicular Zn$^{2+}$ and F$_o$ the intensity in a region with little vesicular Zn$^{2+}$.

**Fluorimetry**

Excitation-emission spectra and fluorescent time-courses were determined on a Hitachi F-4500 spectrofluorimeter in a rapidly stirred methacrylate cuvettes whose temperature was controlled by a circulating water bath (26 °C). Time-courses were determined in a Hepes buffered saline containing (in mM): 140 NaCl, 2.5 KCl and 10 Hepes (pH 7.4). All experiments were performed in 2ml of Hepes saline. Quoted concentrations are all final concentrations in the cuvette or recording chamber. All additions to the cuvette were less than 1% of total volume.
Liposomes were prepared by ultrasonicating 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids) in Hepes buffered saline according to our published procedure (Snitsarev et al. 2001).

Results are reported as Mean ± SEM.

**Reagents**
TPEN (Fluka), ZnAF-2 (Alexis), ZP1(Toronto Research Chemicals) and ZP4 (Neurobiotex).

**Results**

**ZnAF-2 and ZP4 are membrane permeant and load synaptic vesicles**

Acutely prepared rat hippocampal slices were incubated in the presence of ZnAF-2 (5 μM). Within a short time fluorescently labeled terminals became evident in the dentate gyrus and stratum lucidum (Fig. 1 a & b), the fluorescence increased towards a steady-state with a roughly exponential time course with a time constant of 3.3 ± 0.2 min. (n=3, Fig. 2). The co-application of Ca-EDTA (1mM) and ZnAF-2 did not prevent labeling of synaptic terminals suggesting that the probe is not disclosing Zn\(^{2+}\) in the extracellular space but in an intracellular compartment. Addition of TTX did not prevent labeling nor slow its time course implying that neuronal activity is not necessary for internalizing the probe (data not shown).

If ZnAF-2 is present in the extracellular space, the application of exogenous Zn\(^{2+}\) should lead to an elevation of the fluorescence. To test this prediction 2μM of ZnAF-2 was added to a slice preloaded with ZnAF-2 (10 μM) (Fig. 2b). Addition of 1 μM ZnSO\(_4\) to the bath rapidly increased the fluorescence, consistent with the presence of ZnAF-2 in the extracellular space. The slice was then washed for 10 min to remove extracellular ZnAF-2. Although at this point fluorescent puncta were clearly evident in the hilus, now application of 1 μM ZnSO\(_4\) did not increase the fluorescence. Pressure ejection of 10 μM ZnSO\(_4\) into the hilus of a slice washed after preloading with 10 μM ZnAF-2 did not induce an increase in fluorescence (data not shown). All of this provides further support for the hypothesis that most of the fluorescence arises from ZnAF-2 located within an intracellular compartment.

To determine if ZnAF-2 was being taken up by spontaneous endocytosis, slices were incubated with 5 μM ZnAF-2 for one hour at 4 °C to suppress endocytosis (Yang et al. 2005). The fluorescence intensity in the hilus of the dentate gyrus of slices loaded at 4 °C was 21.3±1.2 (n=6) %ΔF/F compared to 29.6±3.6 (n=8) %ΔF/F in control slices held at room temperature for one hour. This suggests that most of the probe loads simply by diffusing across membranes. The reduced loading at 4°C may result from the slowing of diffusion at low temperatures or from suppression of spontaneous endocytosis.
The application of 2 μM ZP4 to hippocampal slices also led to labeling of Zn$^{2+}$-rich terminals (Fig. 1 b&d), with an approximately exponential time course ($\tau = 14.9 \pm 2.1$ min ($n=3$)). Ca-EDTA did not quench the signal but TPEN did ($vita infra$). The fluorescent signal obtained with ZP4 was more intense than ZnAF-2 and the definition of the terminals was sharper. However, ZP4 only stained the outer face of slices, probably because of the restricted diffusion of the probe within the slice. This supposition was supported by the observation that pressure ejecting ZP4 within the depths of a slice led to labeling of terminals close to the electrode tip (Fig. 1e).

**Competition between the Zn$^{2+}$-probes and chelators.**

In order for a chelator to serve as an agent for determining the membrane permeability of a probe it must have a high enough affinity to compete with the probe for Zn$^{2+}$. A competitive assay was performed where chelators were added to Hepes saline containing 1 μM of ZnSO$_4$ and 0.5 μM of the probe. Ca-EDTA (1 mM), EDPA (1 mM) and TPEN (0.2 mM) were able to remove Zn$^{2+}$ from ZnAF-2 (Fig. 3). However, all of these chelators removed Zn$^{2+}$ very slowly from ZP4.

**Permeation of Zn$^{2+}$-probes into liposomes**

To determine if the probes can indeed move passively across membranes a liposomal assay was performed (Snitsarev et al. 2001). The idea is to create a unilamellar vesicle with a high concentration of Zn$^{2+}$ inside and none outside, with the passage of the probe across the membrane being signaled by an increase in fluorescence that is sensitive to the membrane permeant chelator TPEN. Zn$^{2+}$ was entrapped in unilamellar liposomes by sonicating 1,2-dioleoyl-sn-glycero-3-phosphocholine in the presence of 100 μM ZnSO$_4$. The extra-liposomal free-Zn$^{2+}$ concentration was reduced to vanishingly low concentration by the addition of 1 mM EDTA.

The Zn$^{2+}$-probe, FluoZin-3 (Qian et al. 2003), which has three carboxyl groups, was confirmed to be membrane impermeant (Fig. 4). Addition of 0.5 μM FluoZin-3 to liposomes in the absence of EDTA led to a rapid increase in fluorescence as the probe binds to extraliposomal Zn$^{2+}$. Addition of EDTA quickly complexes Zn$^{2+}$ in the extraliposomal compartment. The remaining fluorescence results from the intrinsic fluorescence of the free probe, as TPEN does not quench its fluorescence. Indeed, addition of TPEN elevated the fluorescence. This paradoxical effect might arise if there is some copper or iron bound to the probe that quenches the probes fluorescence, which is then removed by TPEN.

The experiments to determine the permeability of ZP4 and ZnAF-2 were commenced with EDTA in the solution. In all cases the probes crossed the liposomal membrane, as witnessed by the increase in fluorescence that was quenched by the addition of the membrane permeant chelator TPEN (Fig. 4 b&c). The increase of fluorescence induced by the addition of the probe does not arise from the probe abstracting Zn$^{2+}$ from EDTA (see dotted lines in...
Fig. 4 b & c, with only extra-liposomal Zn\(^{2+}\)). ZP4 was found to permeate rapidly across the liposomal membrane whereas ZnAF-2 did so more slowly (Fig. 4).

It should be noted that in the presence of liposomes, TPEN was able to chelate Zn\(^{2+}\) from ZP4 while in Hepes saline it could not. This may result from the presence of other functional groups, forming quaternary complexes with ZP4, Zn\(^{2+}\) and TPEN, dislodging Zn\(^{2+}\) from the probe. Alternatively, partitioning of the Zn\(^{2+}\)-ZP4 complex into the lipid bilayer may lower the affinity of ZP4 for Zn\(^{2+}\).

**Activity dependent increases in ZnAF-2 and ZP4 fluorescence**

As we have shown above, both ZnAF-2 and ZP4 diffuse into synaptic vesicles, therefore, if the slices are stimulated and Zn\(^{2+}\) release occurs the fluorescence intensity should decline as the probe-metal complexes diffuse into the extracellular space; much as in the same way as does the lipophilic probe FM1-43 (Cochilla et al. 1999), although the fluorescence of the latter declines when it partitions into aqueous solution. However, as Ueno et al. (2002) have shown stimulating synaptic pathways in hippocampal slices loaded with ZnAF-2 led to an increase in fluorescence.

Fig. 5 near here

After loading hippocampal slices in 10 \(\mu M\) ZnAF-2 for at least 30 min, transferring them to control saline and washing for 3-5 min, the fluorescence declined as the probe diffused out of the slice. Stimulation of the slices with 50 mM KCl increased the fluorescence intensity, reaching half-maximal levels at about 1 min (Fig. 5). Elevations in fluorescence could be induced by potassium concentrations as low as 10-15 mM.

The simplest hypothesis that could account for the stimulus induced elevation in fluorescence is that traces of extracellular ZnAF-2 persist after washing and detect released Zn\(^{2+}\). However, we showed above that application of Zn\(^{2+}\) to slices preloaded with ZnAF-2 and washed did not induce a response (Fig. 2b). Moreover, washing the slices for 2 hours after loading with ZnAF-2 did not abolish the response to high potassium stimulation. In all experiments where high potassium induced an increase in fluorescence, the elevation was always superimposed on existing puncta of fluorescence (Fig. 5b). If the increase in fluorescence resulted from the release of Zn\(^{2+}\) into the extracellular space and detection by unwashed ZnAF-2 one might expect fluorescent spots to arise in unstained areas, whereas none in fact did.

If during exocytosis Zn\(^{2+}\) is released, the fluorescence should decline as the Zn\(^{2+}\)-probe complex diffuses into the extracellular space and then into the bathing solution. At the end of stimulation the signal should be less than that at the beginning. If stimulated slices are compared to control ones, even after 10 min, the signal is in fact higher in stimulated slices than that in controls.

The dissipation of fluorescence after synaptic activation should be more dramatic while imaging individual synaptic terminals than in low power views of the dentate gyrus, if Zn\(^{2+}\) is released, as the terminals can be seen in isolation from neighboring elements (Gee et al. 2002). Individual synaptic boutons were imaged during the course of stimulation with 50 mM KCl. The intensity of the fluorescent puncta increased and remained elevated for more than 90s, however, no appreciable dissipation of the fluorescence (Fig. 5b & c). The increase in fluorescence between puncta arises from the numerous out-of-focus terminals. Qualitatively similar results held true for ZP4.

Fig. 6 near here.
It could be argued that the metal-probe complex does not diffuse because it might adhere to membranes. To investigate the mobility of the complex in the extracellular space, Zn\(^{2+}\) - ZnAF (100 μM ZnAF-2 & 500 μM ZnSO\(_4\)) was pressure ejected into hippocampal slices and the time course of the fluorescence was monitored. Ejection led to a rapid increase in fluorescence that then dissipated, with little being left after about two minutes (Fig. 6). This experiment suggests that the metal-probe complex appears to diffuse freely in the extracellular space. Using the method of Thorne et al. (2004) the diffusion coefficient was found to be ~3x10\(^{-6}\) cm\(^2\)s\(^{-1}\).

**Cryosections**

Fig. 7 near here.

It has been reported that in cryostat sections of rat hippocampus that ZP4 does not appear to stain synaptic vesicles (Burdette et al. 2003). In light of our findings on acute hippocampal slices we decided to reexamine the permeability of ZP4 in cryosections. In cryostat sections of rat hippocampus we found that ZP4 stained both the hilus and the mossy fibers in a diffuse fashion consistent with distribution of vesicular Zn\(^{2+}\). In slices from animals that had been subjected to kainate-induced seizures the staining of vesicular Zn\(^{2+}\) was dramatically reduced compared to that in control animals (Fig. 7a). The intensity of the vesicular staining as measured by the %ΔF/Fo was for epileptic animals: CA3 (*stratum lucidum*) 0.5 ± 3.0 (n=7) and the Hilus 22.0 ± 3.0 (n=7), while for control animals the %ΔF/Fo were: CA3 37.0±1.0(n=11) and the Hilus 49.0±1.0 (n=13). In control animals only diffuse staining of what are presumed to be synaptic terminals was observed, although individual terminals could not be resolved. In epileptic animals, cells with elevated Zn\(^{2+}\) levels were found scattered throughout the hilus (Fig. 7).

**Discussion**

The significance of the signals emitted by a fluorimetric probe can only be made sense of if the location of the probe can be established with some certainty. In order to pinpoint the Zn\(^{2+}\) probes location we used chelators with known permeability characteristics in conjunction with hippocampal slices and liposomes. The free-acid forms of both both ZnAF-2 and ZP4 were found to be membrane permeant, as was ZP1 (results not shown) (Burdette et al. 2001) Bathing hippocampal slices in solutions containing these probes disclosed the location of Zn\(^{2+}\) rich synaptic vesicles without the need for appending acetoxymethyl (AM) ester groups. Our findings suggest that the permeability characteristics of a probe should be established in the tissue of interest, as the permeability observed in cultured cells may not necessarily extend to brain slices (Frederickson et al. 2004).

We have shown previously that the passage of Zinquin (Zalewski et al. 1994) and TFLZn (Budde et al. 1997) across membranes occurs by simple diffusion and does not depend upon cellular transport (Snitsarev et al. 2001). In spite of the synthesis and availability of an ethyl ester derivative of Zinquin, which although it may increase the rate and degree of loading and may be useful for some cell types, it does not appear to be necessary in order to load hippocampal slices; the same holds true for ZnAF-2 and ZP4.

Cryosections have been widely used to examine tissue stained with TSQ (Frederickson et al. 1987) in unfixed tissue as the synaptic Zn\(^{2+}\) pool appears to be lost.
during fixation. Burdette et al. (2003) found that ZP4 did not stain synaptic vesicles in cryosections. Here, we provide evidence that it does in fact do so, but that vesicular Zn$^{2+}$ staining with ZP4 is diminished in animals subjected to kainate induced seizures.

It is perhaps worth noting that if a probe stains the Zn$^{2+}$ rich tract in the hippocampus, this in itself suggests that the probe is membrane permeant, because Zn$^{2+}$ is found in synaptic vesicles which can only be reached after diffusion across the plasma membrane and thence on into the vesicles. However Zn$^{2+}$, may be loosely associated with macromolecules on the extracellular aspect of cells, forming what has been termed a ‘Zn$^{2+}$-veneer’ (Kay 2003). Vesicular staining can be distinguished from the veneer by the fact that the latter is sensitive to EDPA while the former is not.

Perhaps the simplest and most enduring hypothesis for the existence of synaptic Zn$^{2+}$ is that it serves as a neuromodulator, co-released with glutamate and then acting to modulate sites on the post- or presynaptic membrane. There is much evidence that exogenous Zn$^{2+}$ can modulate a host of channels and proteins, however, only a few papers have provided evidence for synaptically released Zn$^{2+}$ modulating specific postsynaptic sites (Smart et al. 2004).

Fig. 8 near here

If Zn$^{2+}$ is released by exocytosis as a simple hydrated ions, it should spread relatively freely by diffusion, much as does glutamate. The extracellular space is of course chemically and spatially complex, however, reduced models have proved extremely useful in estimating the diffusion of molecules like glutamate in the extracellular space (Barbour and Hausser 1997). Such models based on the physics of diffusion allow one to obtain an estimate of the dynamics of diffusion. A simple model presents itself for estimating the diffusion of molecules in the extracellular space, namely, diffusion of molecules in an infinite planar medium. If M moles are deposited at a point (r=0) at t=0 then the concentration, C(r,t), at a radial distance r from the point source is given by (Crank 1975).

$$C(r,t) = \frac{M}{4\pi h D t} \left( \frac{r^2}{4Dt} \right) e^{-\frac{r^2}{4Dt}}$$  \hspace{1cm} (1)

Where D is the diffusion coefficient and h is the height of the plane (20nm). For the D of Zn$^{2+}$ we will use our value of D=3x10$^{-6}$ cm$^2$s$^{-1}$ for the Zn$^{2+}$-ZnAF-2 complex, which is close to a recently measured value for glutamate in slices (Nielsen et al. 2004), even though the diffusion of Zn$^{2+}$ is likely to be faster because of its smaller hydrodynamic radius. Using ZnT3 knock out mice to estimate the amount of Zn$^{2+}$ in synaptic vesicles (Cole et al. 1999), if we assume that 10% of synaptic vesicles release their contents into the synaptic cleft and if synaptic clefts represents 1% of the extracellular space (Kay 2003; Schikorski and Stevens 1997) the Zn$^{2+}$ concentration in the cleft should be about 5 mM. If we assume that 1 μs after release the concentration of Zn$^{2+}$ in the cleft is 5 mM, the Zn$^{2+}$ concentration will decline to one-thousandths of its initial value within 1 ms (Fig. 8 a&b). Whereas, at a distance of 3μm from the release site the Zn$^{2+}$ concentration will only rise to 5x10$^{-3}$ of its maximum at the release site 10ms after exocytosis (Fig. 8 a). Furthermore, if we assume that an extracellular probe can detect 1nM Zn$^{2+}$ it should be possible to detect an increase in fluorescence 10 μm away from the synapse with a delay of about 10 ms. It should be noted that this simple model will inflate the estimate of both the duration of the Zn$^{2+}$ transient and its diffusion in a 3D
model. Moreover, Zn\(^{2+}\) transporters will accelerate that rate at which Zn\(^{2+}\) is cleared from the extracellular space. Therefore, in a slice the transient changes in extracellular Zn\(^{2+}\) might be expected to be more fleeting than the estimates from planar diffusion.

What are we to make of the increases in fluorescence induced by stimulation that both we and Ueno et al. (2002) observed in slices loaded with ZnAF-2? Three aspects of this phenomenon stand in contradiction to the notion of Zn\(^{2+}\) release. Firstly, if a Zn\(^{2+}\)-ZnAF-2 complex were being released from the vesicles it should lead to a decline in the vesicular fluorescence as the complex diffuses out of the vesicle. Secondly, the increase in fluorescence induced by stimulation endures for many minutes. Whereas diffusion would again predict that it should dissipate within a few milliseconds. Thirdly, the increase in fluorescence occurs in register with existing areas of fluorescence. While release would predict increases in fluorescence shifted away from boutons.

As regards the first point, the increase in fluorescence could result from the rise in intravesicular pH (Sankaranarayanan et al. 2000) as it undergoes exocytosis, a change in the molecular environment or dequenching of the probes. However, in both cases the release of Zn\(^{2+}\) should ultimately lead to the fluorescence falling below control levels.

We have suggested that the zinc in synaptic vesicles is not freely diffusible but appears to be in association with molecules on the membrane (Kay 2003). We have argued that vesicular Zn\(^{2+}\) is unable to diffuse into the extracellular space on exocytosis because it is tethered to a membrane associated macromolecule through two to three coordination bonds (Fig. 8). Under this scenario the Zn\(^{2+}\) is what we term ‘externalized’. This presents the experimentalist hoping to show release with a fluorimetric probe with something of a conundrum because if a probe is present in the extracellular space it is likely to abstract Zn\(^{2+}\) from membrane associated coordination site. This will then give rise to an apparent release event, resulting entirely from the presence of the probe. There does not seem to us to be any way of distinguishing apparent release from de facto release using diffusible Zn\(^{2+}\)-probes, one has to resort to analytical methods like Inductively Coupled Plasma Mass Spectrometry (Taylor 2000) or a Zn\(^{2+}\) electrode, if one with high enough sensitivity were available.

The externalization of Zn\(^{2+}\) is compatible with our results on both FluoZin-3 (Kay 2003) and ZnAF-2. It is also consistent with the results of Qian and Noebels (2005) who found with FluoZin-3 that there was a 50 ms latency between the peak of the epsp and the transient response of FluoZin-3. If Zn\(^{2+}\) were released in free form, because FluoZin-3 binds Zn\(^{2+}\) very rapidly, no latency should be expected. However, if the probe is competing with the membrane binding site a delay might be expected. In the case of ZnAF-2 it appears that the probe forms a ternary complex with the membrane macromolecule through the intermediary of Zn\(^{2+}\) and that this complex remains attached to the membrane. The formation of ternary Zn\(^{2+}\) complexes is well established in biology and chemistry (Hendrickson et al. 2003).

Our results suggest that the Zn\(^{2+}\) in synaptic vesicles is unlikely to be in free-form, but that it appears to be complexed with an as yet unidentified macromolecule. The results presented here are at odds with the longstanding hypothesis of Zn\(^{2+}\) release (Assaf and Chung 1984; Howell et al. 1984). Only the judicious use of Zn\(^{2+}\) probes and physical techniques coupled with an understanding of the cellular terrain is likely to resolve the question of synaptic Zn\(^{2+}\) mobilization and its role.
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Figure Legends

Fig. 1 Loading of slices with Zn^{2+}-probes.
Hippocampal slices loaded with ZnAF-2 (a&c) and ZP4 (b&d). Scale bars; 250 μm a&b and 10 μm c&d. (e) An image approximately 50 μm below the surface of a hippocampal slice where 10 μM of ZP4 was pressure ejected into the stratum lucidum. The shank of the electrode is visible on the right hand edge of the image. Four 500 ms pressure pulses (18 psi) were delivered from an electrode with a tip diameter of approximately 1 μm. Scale bar 20 μm.
Fig. 2 ZnAF-2 loads synaptic vesicles in live hippocampal slices.
(a) The time course of loading of hippocampal slices with ZnAF-2 (5μM). The fluorescence was measured in the hilus (n=3) and was normalized to the maximum value during the experiment. (b) The fluorescence of a hippocampal slices with extracellular ZnAF-2 increased when Zn$^{2+}$ was applied, but not after ZnAF-2 was washed off. The slice was loaded with 10 μM ZnAF-2 for one hour prior to the experiment.
Fig. 3 Responses of ZP4 and ZnAF-2 to the application of chelators. The experiments were carried out in Hepes saline with 2 mM CaCl$_2$ and 1.3 mM MgSO$_4$. (a) ZnAF-2 (b) ZP4.
**Fig. 4** Passage of Zn$^{2+}$-probes across liposomes.

Liposomes with Zn$^{2+}$ trapped inside and no Zn$^{2+}$ outside, were used to determine if Zn$^{2+}$ probes could cross lipid bilayers passively (a) Fluozin-3 was added to a liposomal solution with Zn$^{2+}$ (100 μM) inside and outside of the liposomes. EDTA was then added to remove the Zn$^{2+}$ in the medium. (b & c) Determination of the permeability of ZP4 and ZnAF-2 respectively, experiments were commenced with EDTA (1mM) in the liposomal solution. The solid line indicates experiments in which the liposomes were prepared with 100 μM ZnSO$_4$, while the dotted line represents liposomes prepared without ZnSO$_4$ but with 10 μM ZnSO$_4$ added just before commencing the experiment. Experiments were carried out in Hepes saline at 26 °C.
Fig. 5 KCl stimulation induces an increase in fluorescence in hippocampal slices but the elevations do not spread.

(a) Slices were loaded with 10 μM ZnAF-2 for at least 1 hr. The fluorescence was measured in the hilus. Slices were stimulated by the application of 50 mM KCl to the bath which was then washed off after 15s (●, n=4) or 15 mM KCl for 30s (●, n=3). Control slices were not stimulated (∗, n=5). (b) Pseudo-color image of terminals in the dentate gyrus prior to stimulation. Scale bar 5 μm. (c) Pseudo-color plot of the intensity along the dotted line in ‘b’ during the application of 50 mM KCl (●). The green trace show how the fluorescent intensity changes in the brightest terminal in the lower right hand corner of ‘b’. The total time represented is 90s. The color scale runs from 830-910.
Fig. 6 ZnAF-2 diffuses freely in the extracellular space. A solution containing 100 μM ZnAF-2 and 500 μM ZnSO₄ was pressure ejected (100ms & 18 psi, electrode tip diameter ~ 1 μm) into the stratum radiatum of CA1 about 50 μm below the surface of the slice. (a) Pseudo-color image 6 s after pressure ejection. Scale bar 100μm. (b) Pseudo-color time-scan along the vertical line in ‘a’. (c) Fluorescence intensity as a function of time along the horizontal line in ‘c’. Time scale bar 30s. Color scale bar applies to all panels and runs from 552 to 821.
Fig. 7 Seizures reduce vesicular staining in cryosections
(a) ZP4 stained cryosections of the hippocampus from control and epileptic rats. Scale bar 50 μm. h – hilus, sl – stratum lucidum, so – stratum oriens, sp – stratum pyramidale & sr – stratum radiatum. (b) The hilus of a ZP4 stained slice from an epileptic rat that was sectioned 18 hours after kainate injection. Slices were incubated in 5μM ZP4 dissolved in ACSF for 30 minutes. The granule cell layer is evident in the lower right hand corner. Scale bar 100 μm.
**Fig. 8 Diffusion of Zn$^{2+}$ in the extracellular space.** Diffusion in an infinite planar medium calculated from equation 1. $D = 3 \times 10^{-6} \text{ cm}^2 \text{s}^{-1}$. Zn$^{2+}$ concentration is normalized by the concentration at $r=0$ after 1 $\mu$s. Since the equation is linear the solutions can be scaled to an arbitrary initial value. (a) Normalized $[\text{Zn}^{2+}]$ as a function of time at different distances from $r=0$. (b) Normalized $[\text{Zn}^{2+}]$ as a function of $r$, at different times after release. (c) A schematic distinguishing release from externalization.
References


