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The zinc indicator FluoZin-3 is not perturbed significantly by physiological levels of calcium or magnesium

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Abstract

There has been some dispute in the literature as to the sensitivity of the zinc indicator FluoZin-3 to calcium, with suggestions that physiological levels of calcium and magnesium effectively occlude the response of the probe to zinc. In this communication we demonstrate that calcium concentrations as high as 10 mM do not prevent FluoZin-3 from detecting zinc elevations as low as 1 nM. Moreover, the inclusion of a few μ M Ca-EDTA does not prevent FluoZin-3 from responding to increases in zinc concentration but does extend the dynamic range of the probe by reducing contaminating zinc levels and allowing the probe to respond to multiple zinc additions. In addition, we have derived a mathematical model to account for the kinetics of FluoZin-3 response to zinc in the presence of an additional zinc and calcium chelator.

Keywords

zinc; fluorescence; indicator; probe

Introduction

Zinc in tight association with a variety of proteins plays an important role in the life of cells as a structural or catalytic element [1]. Calcium which enters into looser associations with proteins acts as a second messenger molecule, initiating a number of cellular processes when its 'free' concentration is elevated intracellularly. There are some indications that zinc may serve a similar role, however, because the free zinc concentration appears to be held at levels a few orders of magnitude below that of calcium, it has been far more difficult to demonstrate this role conclusively [2]. Fluorescent probes have played an invaluable role in demonstrating and visualizing the role of calcium as a second messenger. In the last few years there has been considerable activity in the development of zinc probes [3], with the hope that such indicators may disclose whether free zinc does indeed serve as a cellular messenger.

A number of zinc sensitive fluorescent probes with a variety of different zinc binding moieties and fluophores have been developed. From a cell biological perspective these indicators can

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be divided into two categories; those that cross membranes and those that do not [4]. Membrane permeant probes such as TFLZn [5], Zinquin [6], ZnAF-2 [7], ZP1 [8] and ZP4 [9] have proved useful in exploring the zinc-rich vesicles found in a number of cell types. In contrast, membrane impermeant probes can be used to visualize zinc in the extracellular space [10,11]. Of these the one that has perhaps the firmest credentials as an impermeant probe is FluoZin-3 (registered trademark of Molecular Probes Inc.), which has been successfully used to detect zinc release from pancreatic β -cells [10] and what has been termed the 'externalization' of zinc from hippocampal slices [11], as well as measuring the affinity of the metallothionein zinc-binding sites [12]. FluoZin-3, in the form of an acetoxymethyl (AM) ester derivative, has also proved useful in a variety of biological preparations for detecting intracellular zinc elevations [13-19].

In contrast to the simplicity of defined aqueous media, the complex heterogeneous environment within a cell complicates the interpretation of the changes in fluorescence of indicator molecules [4]. Moreover, zinc indicators in the extracellular space have the additional challenge of functioning in a milieu where the concentration of calcium and magnesium are many orders of magnitude higher than zinc. These factors present pitfalls to the investigator attempting to make sense of fluorescent signals emanating from zinc indicators within the confines of biological tissue.

In this communication we present data on the interaction of FluoZin-3 with zinc, in the presence of chelators, calcium and magnesium.

Materials and methods

Fluorimetry

Excitation-emission spectra and fluorescent time-courses were determined on a Hitachi F-4500 spectrofluorimeter using a stirred methacrylate cuvette (Fisher Scientific) whose temperature was controlled by a circulating water bath at 26 °C. The monochromator slit widths were set at 5 nm and the data was sampled at 1 Hz. All experiments were performed in a Hepes buffered saline containing (in mM): 140 NaCl, 2.5 KCl and 10 Hepes (pH 7.4) with FluoZin-3 at a concentration of 500 nM unless otherwise specified. The excitation wavelength was set at 494 nm and the emission at 518 nm. All additions to the cuvette were made with metal-free pipette tips (Fisher Scientific).

Reagents

N,N,N',N'-Tetrakis-(2-pyridylmethyl)-Ethylenediamine (TPEN, Fluka), Hepes (Calbiochem) and FluoZin-3, tetrapotassium salt (Molecular Probes Invitrogen). The following prepared solutions were used: 1 M CaCl_2 (Fluka, 2115), 1M MgCl_2 (Sigma, M-1028) and 51 mM ZnSO_4 (Aldrich, 31,962-7). All other reagents were from Sigma.

Results

FluoZin-3 exhibits a single excitation peak at 494 nm and an emission peak at 518 nm, neither of which shift on the addition zinc or any other biological transition metals. Little contaminating metal was found in the analysis of a commercial sample of FluoZin-3. As we have shown previously on addition of the probe to a methacrylate cuvette the fluorescence increases as a result of zinc being leached from the cuvette and stir bar, that is accelerated by stirring [20].

During the course of these experiments we found that FluoZin-3 appears to breakdown in distilled and deionized water at 4-6°C, becoming less responsive to zinc when held for extended periods of time. For example a stock solution of 1mM FluoZin-3 held for 17 months at ~4°C showed only a 3.1 times elevation on the addition of 1 μM zinc compared to 14.1 times in fresh

probe. LC-MS analysis of the aged solutions revealed that FluoZin-3 slowly decomposes into lower molecular weight species. The mass of the predominant breakdown product corresponds to loss of a CH_2CO_2 fragment, resulting in a N,N- or N,N'-bis-acetic acid derivative. This hypothesis was validated by comparison of the zinc response and co-elution with an authentic bis-acetic acid sample prepared independently by synthesis (data not shown). It is not known which N-acetic acid residue is lost upon decomposition, but there are precedents for substantially lowered ion affinities upon loss of an N-acetic acid fragment(s) from fluorescent ion sensors based on the BAPTA chelator [21]. It is recommended that aliquots of solution be frozen down to -20°C to increase the lifetime of the probe.

In a number of recent publications it was suggested that the presence of calcium at levels typically found in the extracellular space, prevents FluoZin-3 from responding to the addition of zinc [22-25]. The authors proposed that FluoZin-3 is saturated in the presence of 2 mM calcium, so that the application of zinc cannot further boost the fluorescence. To test this assertion 1 μM zinc was added to a solution with physiological levels of calcium and magnesium. Addition of 2mM calcium or magnesium led to only small increases in the fluorescence of FluoZin-3 ($\sim 11\%$, $\sim 7\%$ respectively), whereas addition of 1 μM zinc led to a $\sim 1562\%$ increase (Figure 1 a). Contrary to the claims mentioned above, the presence of 2mM calcium and 2mM magnesium did not abolish the response of the probe to the addition of a two thousand-fold lower concentration of zinc (1 μM) but did reduce it by about $\sim 25\%$.

Part of the response to calcium or magnesium may result from contaminating zinc in the stock solutions. This is shown in Figure 1 b where the addition of the transition metal chelator TPEN [26] (10 μM) after 10 mM calcium had been added, quenched the signal by $\sim 57\%$. The larger response of FluoZin-3 to calcium (20-30% of the response to zinc) observed by Martin et al. as compared to $\sim 0.7\%$ in this study suggests that their calcium stock solutions may be more severely contaminated with zinc. Although TPEN has a low affinity for calcium the concentration employed in this experiment, is too low to substantially alter the free calcium concentration.

Combining FluoZin-3 with Ca-EDTA

When zinc is added to a solution containing Ca-EDTA and FluoZin-3 in the presence of excess calcium, the zinc binds first to the probe and then is slowly abstracted away by the EDTA [11]. Indeed so slow is this, that the initial fluorescence elicited by the addition of zinc is no different in the presence or absence of Ca-EDTA (Figure 2a). Addition of low concentrations of Ca-EDTA do not perturb the response of FluoZin-3. In fact, because it decreases the background levels of zinc, it extends the dynamic range of the probe. Moreover, because Ca-EDTA eventually binds added zinc, it makes the probe reversible, albeit with a long time-constant (Figure 2a). We have previously shown in cuvette experiments that FluoZin-3 with 10 μM Ca-EDTA can detect zinc additions as low as 100 pM [20].

In what follows we have attempted to account for the biphasic response of FluoZin-3 in the presence of Ca-EDTA in terms of the known thermodynamic and kinetic characteristics of EDTA and FluoZin-3. From the dissociation constants for zinc and calcium with EDTA (0.1 nM and 263 μM respectively) [27] in a solution with 500 nM FluoZin-3 and 50 μM Ca-EDTA, with 2mM CaCl_2 , the free EDTA concentration is estimated to be ~ 2.5 pM and that of FluoZin-3 ~ 58 nM. Because of the $\sim 23,000$ fold higher concentration of FluoZin-3 compared to EDTA, added zinc will bind rapidly to FluoZin-3, giving rise to a fast increase in fluorescence. The near instantaneous increase is followed by a monotonic decline (Figure 1b). FluoZin-3 cannot abstract zinc from EDTA because of its thermodynamic disadvantage. The decline of fluorescence essentially arises from the free EDTA binding free zinc, which proceeds slowly because both are at low concentrations.

To derive equations that characterize the FluoZin-3, EDTA, zinc and calcium equilibrium we make the assumption that zinc binds rapidly to FluoZin-3 (F) and then is appropriated by EDTA as governed by the following equilibria:

If we make the steady-state approximation on the zinc concentration (i.e. $d[Zn]/dt = 0$) we can then derive the following equation that predicts how fast EDTA sequesters zinc from FluoZin-3 (see appendix).

$$\left[1 + \frac{k_1 F_t}{B} \right] \ln \left[\frac{C_F}{C_{F0}} \right] + \frac{k_1}{B} (C_{F0} - C_F) = -k_{-1} t \quad 5$$

Where: $B = k_2 [EDTA](1 + K_{a4}[Ca])$, $C_{F0} = [F \cdot Zn]$ at $t=0$, $C_F = [F \cdot Zn]$ at t and F_t = total FluoZin-3 concentration and K_{a4} is the association constant for the formation of $F \cdot Ca$.

Equation 5 provides a good fit to the changes in fluorescence observed on the addition of zinc and consistent estimates of k_1 , k_{-1} and k_2 (Figure 2b, Table 1).

Discussion

The high prevailing levels of calcium and magnesium in the extracellular solution surrounding cells provides a challenge to detecting transient elevations of zinc. FluoZin-3 appears to be well matched to this task because of its low affinity for calcium and magnesium. It is worth noting that the first use of FluoZin-3, which was to detect zinc release from pancreatic β -cells, was made in the presence of 2.4 Ca and 1.4 Mg [10]. We have shown here that paradoxically the provision of low levels of Ca-EDTA improves the dynamic range of FluoZin-3 by reducing contaminating zinc. It also has the additional advantage that it in effect resets the indicator allowing it to register repeated elevations in zinc levels.

Martin et al. [22] made the claim that elevated calcium levels essentially occlude the response of FluoZin-3 and conclude, "Therefore, a probe like FluoZin-3 is not an optimal choice for probing Zn in an environment in which high concentrations of Ca are present, such as the extracellular space." Our experiments shown in figure 1a demonstrate rather clearly that even in the presence of high calcium and magnesium, FluoZin-3 remains responsive to zinc elevations.

Martin et al. [22] make a further claim that should be addressed, namely, "The inability of FluoZin-3 and Zinpyr-4 to detect Zn at low μM concentrations limits their application in biological systems where the background Zn level is close to the concentration at which they become saturated." In experiments with cells or brain slices there may indeed be levels of zinc sufficient to bind to FluoZin-3 [11], however, we have shown that the provision of a low concentration of Ca-EDTA mops up contaminating zinc while not affecting the responsiveness of the probe.

Some of the infelicities of Martin et al. [22] have been addressed by Dineley [28] in a letter to *Cell Calcium*. We thought that it might be of some value to experimentally assay FluoZin-3's calcium and zinc sensitivity, to put this contentious issue to rest. It is perhaps worth bearing in mind that although one may take every precaution to rid solutions of zinc contamination, the completeness of one's efforts should be tested by the addition of TPEN. For example, if the addition of a nominally pure calcium solution leads to the increase in fluorescence of an indicator, the addition of TPEN should be without effect if calcium alone is responsible for the change.

Some of the disparities between our work and those of others may have resulted from differences in the breakdown of the molecule, however, they do not appear to have examined the effect of the sequential addition of calcium, magnesium and then zinc on the fluorescence of FluoZin-3 (Figure 1a). This maneuver is essential if one is to prove that calcium or magnesium blunts the sensitivity of a probe.

The panoply of new zinc indicators presents a wonderful opportunity for biologists interested in exploring the role of zinc in cell biology. However, without rigorous tests, both *in vivo* and *in vitro*, of their characteristics, their promise will remain unrealized and they may serve as agents of confusion rather than enlightenment.

Appendix

Competition between EDTA and FluoZin-3 for calcium and zinc

Considering equilibria 1-4, if the total fluophore concentration is:

$$F_t = [F \cdot Zn] + [F] + [F \cdot Ca] \quad (A1)$$

$$K_{a4} = \frac{[F \cdot Ca]}{[F][Ca]} \quad (A2)$$

$$\frac{d[Zn]}{dt} = -k_2[Zn][EDTA] + k_{-1}[F \cdot Zn] - k_1[F][Zn] \quad (A3)$$

$$\frac{d[F \cdot Zn]}{dt} = -k_{-1}[F \cdot Zn] + k_1[F][Zn] \quad (A4)$$

Substitute A2 in A1

$$[F] = \frac{F_t - [F \cdot Zn]}{1 + K_{a4}[Ca]} \quad (A5)$$

If we make the steady-state approximation on [Zn] and then substitute A5 into A3:

$$[Zn] = \frac{k_{-1}[F \cdot Zn]}{k_2[EDTA] + k_1[F]} \quad (A6)$$

$$\frac{d[F \cdot Zn]}{dt} = \frac{-k_{-1}k_1[F \cdot Zn][EDTA]}{k_1[EDTA] + k_1[F]} \quad (A7)$$

Separating variables:

$$\frac{d[F \cdot Zn]}{[F \cdot Zn]} \left[1 + \frac{k_1[F]}{k_2[EDTA]} \right] = -k_{-1}dt \quad (A8)$$

Equation A6 is substituted into equation A8, then:

$$\frac{d[F \cdot Zn]}{[F \cdot Zn]} \left[1 + \frac{k_1 F_t}{B} \right] - \frac{k_1}{B} d[F \cdot Zn] = -k_{-1}dt \quad (A9)$$

where: $B = k_2 [EDTA](1 + K_{a4}[Ca])$

The following boundary condition are used to resolve equation A9: when reaction time = 0, $[F \cdot Zn] = C_{F0}$; when reaction time = t, $[F \cdot Zn] = C_F$. After integration on both sides:

$$\left[1 + \frac{k_1 F_t}{B} \right] \ln \left[\frac{C_F}{C_{F0}} \right] + \frac{k_1}{B} (C_{F0} - C_F) = -k_{-1}t \quad (A10)$$

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Figure 1.

Effect of calcium and magnesium on the response of FluoZin-3 to zinc. (a) Calcium and magnesium do not prevent FluoZin-3 from responding to zinc. Additions to the cuvette are indicated by the arrows. Neither calcium nor magnesium were added to the experiment represented by the solid black line. (b) Some of the fluorescence that arises from the addition of calcium to FluoZin-3 results from contaminating zinc. Zinc was only added to the sample specified with the gray dashed line.

Figure 2.

(a) FluoZin-3 in the presence of Ca-EDTA and excess calcium and magnesium responds to the application of zinc. The solid black line corresponds to experiment in the absence of Ca-EDTA and dashed gray line in its presences. Both recordings in the presence of 2 mM CaCl_2 , 1.3 mM MgSO_4 (b) Responses of FluoZin-3 (500 nM) to zinc in the presence of different concentration of Ca-EDTA (• 50 μM Ca-EDTA, 0.32 μM Zn; ◦ 50 μM Ca-EDTA, 1 μM Zn; ▼ 10 μM Ca-EDTA, 0.1 μM Zn and Δ 10 μM Ca-EDTA, 0.32 μM Zn). Gray lines represent fits to equation 5, where f = fluorescent intensity $\propto C_F$ and f_0 = maximum fluorescent intensity on the addition zinc $\propto C_{F0}$.

Table 1

Estimates of the rate constants for the interaction of zinc with FluoZin-3 and EDTA

	50 μM Ca-EDTA, 0.32 μM Zn	50 μM Ca-EDTA, 1.0 μM Zn	10 μM Ca-EDTA, 0.10 μM Zn	10 μM Ca-EDTA, 0.32 μM Zn
k_{-1} (s^{-1})	0.055	0.048	0.049	0.042
k_1 (10^6 M. s^{-1})	3.67	3.2	3.3	2.8
k_2 (10^8 M. s^{-1})	1.6	1.98	2.1	2.2