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SHORT COMMUNICATION

Validation of TPEN as a Zinc Chelator in Fluorescence Probing of Calcium in Cells with the Indicator Fura-2

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Abstract Fura-2 is widely used as a fluorescent probe to monitor dynamic changes in cytosolic free calcium in cells, where Ca^{2+} can enter through several types of voltage-operated or ligand-gated channels. However, Fura-2 is also sensitive to other metal ions, such as zinc, which may be involved in ionic channels and receptors. There is interest, in particular, in studying the synapses between mossy fibers and CA3 pyramidal cells which contain both calcium and high quantities of free or loosely bound zinc. We have found, through fluorescence probing, that endogenous zinc inhibits mossy fiber calcium transients. However, since these results might be explained by an effect of the zinc chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) on the spectral properties of Fura-2, we have carried out a validation of the method through fluorescence excitation spectra of the complex Fura-2/calcium, and show that TPEN does not affect these spectra. This supports the idea that the observed calcium enhancement is related to a zinc inhibition of presynaptic calcium mechanisms, and confirms the use of the chelator TPEN as a general procedure for the biophysical study of Ca(II) in the presence of Zn(II) using Fura-2.

Keywords Fluorescence probing · Cellular calcium sensing · TPEN zinc chelator · Fura-2

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Introduction

Fluorescent indicators are valuable tools for studies of ionic influxes in biological preparations, including neurons. Fura-2 is the most widely used fluorescent probe to monitor dynamic changes in cytosolic free calcium in living cells [1–3], although a number of other systems are under development for this [4–6]. Its main advantages are its high quantum yield, leading to a strong fluorescence, its wavelength sensitivity to Ca^{2+} and its relatively high selectivity for this metal ion [1]. However, it has limitations as a calcium indicator, including photobleaching, sequestration in non-cytoplasmic compartments and quenching of Fura-2 fluorescence by heavy metals, such as zinc [2, 7]. The neurobiological importance of Zn^{2+} is increasingly being recognized [8], since the finding that histochemically reactive zinc is present in high concentrations in synaptic vesicles of the hippocampal mossy fibers [9, 10]. Zinc ions, released during neuronal activity [11–13], modulate the action of multiple receptors and channels [14], and may have a neuromodulatory role following intense stimulation [15]. It is, therefore, important to have a selective technique

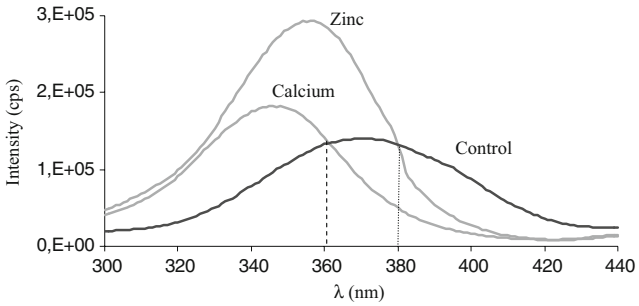

Fig. 1 Fluorescence excitation spectra of Fura-2 and its complexes with calcium and zinc, at t=22 °C and pH=7.15. The emission was collected at 510 nm. Fura-2/calcium and zinc curves were recorded using solutions containing 120 mM KCl, 10 mM MOPS, 1 mM Fura-2, and saturating concentrations of $CaCl_2$ (1 mM) or $ZnCl_2$ (50 mM), respectively. The control curve was obtained using a free calcium and zinc solution, containing EGTA (10 mM) and EDTA (10 mM)

for fluorescence probing of cellular calcium in the presence of zinc. The effect of zinc can be removed by the application of heavy metal chelators, such as the permeant complexant N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) [16, 17], and we have found that the application of TPEN increased presynaptic calcium transients, as seen by Fura-2 fluorescence, without affecting synaptic transmission [18]. It is believed that this results from the blockade of the inhibitory effect of zinc [18], which could be due to the blockage of presynaptic voltage-gated calcium channels [14, 19–21] and/or to the activation of presynaptic K_{ATP} channels [22]. However, the observed dynamic changes in calcium with Fura-2 [18], were seen using fixed wavelengths, selected by narrow band filters for excitation and observation of fluorescence, and an alternative explanation of these observations is that TPEN may modify the spectral properties of the complex Fura-2/calcium. Distinction between these explanations is of obvious biophysical significance both for mossy fiber studies and for other biologically relevant systems, so that we have carried out a fluorescence spectral study on the system to test this second hypothesis.

It has been shown that the complex Fura-2/zinc produces a shift in emitted fluorescence similar to the Fura-2/calcium complex [1]. However, the isosbestic points are different for the two complexes [7], which make the measurement of Fura-2/calcium fluorescence changes possible without significant contamination of zinc. For this purpose, the excitation wavelength for fluorescence measurements of Fura-2/calcium must be as close as possible to the isosbestic point of Fura-2/zinc (380 nm [7]). In our experiments, the excitation wavelength was selected by means of the appropriate narrow-band filter (10 nm bandwidth [18]). To validate our observation, we report a study of the spectra of solutions of the complexes Fura-2/calcium and Fura-2/zinc, of the control solution (ion-free fura-2), and of these systems in the presence of TPEN.

Experimental

Materials

Fura-2, TPEN (Molecular Probes Europe BV, Leiden, NL), ethyleneglycol tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), 3-*N*-morphilinopropionic acid (MOPS) (Sigma-Aldrich) and KCl (Merck) were of the purest grades available, and were used as received. All solutions were prepared in Millipore purified water (resistivity of 18 MOhm cm).

Methods

Fura-2 calibrations were performed using aqueous solutions at 22 °C, buffered with 10 mM MOPS (pH 7.15), and containing 120 mM KCl, 1 mM Fura-2, and selected concentrations of $CaCl_2$ (between 10 μM and 1 mM) or $ZnCl_2$ (1 mM to 50 mM). The calcium and zinc free solutions were obtained adding EGTA (10 mM) and EDTA (10 mM), respectively. The calcium and zinc concentrations were estimated by iteratively solving the non-linear set of equilibrium binding equations describing calcium and zinc binding to the added ligands using published dissociation constants, i.e. 245 nM for calcium/Fura and 3 nM for zinc/Fura complexes. [1, 23]. The heavy metal chelator TPEN was used at a concentration of 100 μM.

Fluorescence excitation spectra were obtained on a Spex Fluorolog 111 spectrofluorimeter in the wavelength range 250–500 nm, with observation of the emission at 510 nm,

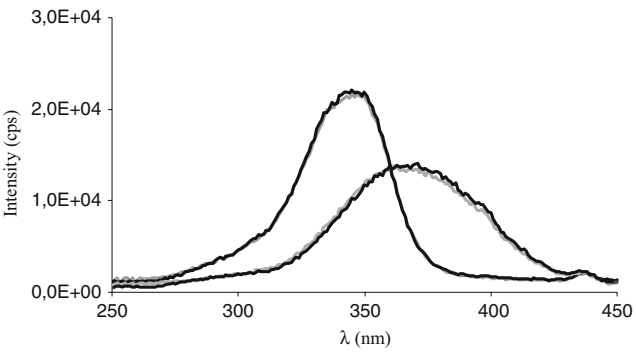

Fig. 2 Fluorescence excitation spectra of Fura-2/calcium complex (left curves) and of Fura-2 with EGTA and EDTA, (right curves) in the absence (dark traces) and in the presence (light traces) of the zinc chelator TPEN (100 μM), at t=22 °C and pH=7.15. The emission spectra were collected at 510 nm

2.5 mm excitation and 1.25 mm emission double slits, 1 nm wavelength increments and an acquisition time of 1 second/point to optimise signal/noise ratio. Data analysis was performed by converting the records into ASCII files and then using Microsoft Excel for graphic and statistic treatments.

Results and discussion

The fluorescence excitation spectra of Fura-2/zinc and Fura-2/calcium complexes were recorded and compared with that of Fura-2 in the absence of both ions, as shown in Fig. 1.

All of the curves are bell-shaped, and both ions shift the fluorescence maximum to the ultraviolet. The isosbestic (or to be more correct the isoemissive) point, where the fluorescence excitation intensity of the ion-free Fura-2 solution is equal to that of Fura-2/calcium (or zinc)-containing solution, is close to 360 nm and 380 nm for calcium and zinc, respectively.

To study the effect of TPEN on the fluorescence excitation spectra of the complex Fura-2/calcium, this zinc chelator was added to the Fura-2/calcium complex, and also to a Fura-2/EGTA solution (free of calcium). Excitation spectra were then measured, to compare the curves in the absence and in the presence of TPEN. The results are presented in Fig. 2. As can be seen in the graphs, TPEN did not change the fluorescence excitation spectra of free or calcium-bound Fura-2.

It was previously observed that hippocampal mossy fiber calcium changes associated with zinc release, determined using Fura-2, increase by approximately 20% in the presence of the permeant zinc chelator TPEN [18].These results may be due to the suggested effects of zinc in presynaptic channels, or may arise from the effects of the zinc chelator TPEN on spectral properties of the fura-2 /calcium complex. The results obtained from the present experiments show that the second explanation is not tenable. We have shown that the isosbestic point of the Fura-2/zinc complex was near the wavelength used for the detection of presynaptic calcium transients (i.e., 380 nm), as previously observed [7,23]. In addition, TPEN does not change the spectral properties of the free or the calcium-bound forms of Fura-2. It has previously been observed in hepatocytes and pancreatic acinar cells that TPEN enhances Fura-2 fluorescence, even at the isosbestic wavelength, but only for cells that have been lightly loaded with this indicator, with the effect being reversed on increasing the concentration of Fura-2 [24]. It has also been shown that, in pituitary and glial cells, TPEN does not affect Fura-2 fluorescence [25]. These results strongly support the suggestion [18] that the observed mossy fiber calcium enhancement is not due to an action of TPEN on Fura-2 spectral properties, but is related to inhibition of presynaptic calcium mechanisms, due to the action of zinc, which may modulate synaptic transmission, especially during intense stimulation.

Conclusions

The zinc(II) chelator TPEN has no effect on the isosbestic point in the fluorescence excitation spectra of Fura-2/calcium fluorescence, and can therefore be used in real-time monitoring of intracellular calcium(II) changes in the presence of zinc(II).

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