Fine structural detection of calcium ions by photoconversion

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Abstract

We propose a tool for a rapid high-resolution detection of calcium ions which can be used in parallel with other techniques. We have applied a new approach by photo-oxidation of diaminobenzidine in presence of the emission of an excited fluorochrome specific for calcium detection. This method combines the selectivity of available fluorophores to the high spatial resolution offered by transmission electron microscopy to detect fluorescing molecules even when present in low amounts in membrane-bounded organelles. We show in this paper that Mag-Fura 2 photoconversion via diaminobenzidine oxidation is an efficient way for localizing Ca2+ ions at electron microscopy level, is easily carried out and reproducible, and can be obtained on a good amount of cells, since the exposure in our conditions is not limited to the direct irradiation of the sample via an objective but obtained with a germicide lamp. The end product is sufficiently electron dense to be detected clearly when present in sufficient amount within a membrane boundary.

Introduction

The ultrastructural localization of a molecule requires that a certain degree of electron-density, resulting in adequate electron scattering, be present in sufficient amount. Binding of heavy atoms can be accomplished easily with regards to proteins, nucleic acids and other cellular components, in some cases with a high degree of specificity. However, the subcellular localization of ions is a challenge, since in many cases ions are diffuse and not easily kept in place after the procedures of sample preparation for electron microscopy (EM). Many ions can offer enough scattering in spite of not being heavy metals, such as manganese, whereas others necessitate of blocking or precipitating techniques to be visualized. In particular, calcium ions are easily diffusible inside the cell, and even when protein-bound are not easily detectable. The pioneering paper by Tandler et al.7 demonstrated the presence of calcium ions by EM via pyroantimonate precipitation, with a good yield of the final product. The end product of the reaction, however, is probably overrated, since the technique is capable of stabilizing and precipitating calcium and other bivalent cations, such as magnesium, as proved by electron spectroscopic imaging (ESI) analysis.8 Since their introduction by Tsien and coworkers,9 calcium studies have used specific Ca2+-sensitive fluorochromes, such as Fura-2, Indo-1 and Fluo-4, coupled to wide-field digitized video fluorescence microfluorimetry and confocal microscopy to image acute elevations in intracellular Ca2+ concentration ([Ca2+]i) with high spatio-temporal resolution.6,10 Cytoplasmic and nuclear Ca2+ signals regulate a multitude of vital cellular processes, such as gene transcription, cell cycle progression, cytoskeletal and chromatin remodelling, and migration.11,12 However, proper Ca2+ handling within the endoplasmic reticulum (ER), the most abundant intracellular Ca2+ reservoir,10 is also crucial for cell-fate decisions. The steady-state ER Ca2+ level ([Ca2+]ER) does not only control synthesis, folding, export and trafficking of proteins,10,11 but it also fuels mitochondrial bioenergetics thereby suppressing autophagy.12 Low affinity Ca2+ indicators, such as Mag-Fura 2, and genetically-encoded probes, such as the bioluminescent protein aequorin and the fluorescence proteins camgaroos, pericams, Indo-1 and Fluo-4, coupled to wide-field digitized video fluorescence microfluorimetry and confocal microscopy to image acute elevations in intracellular Ca2+ concentration ([Ca2+]i) with high spatio-temporal resolution.6,10 Cytoplasmic and nuclear Ca2+ signals regulate a multitude of vital cellular processes, such as gene transcription, cell cycle progression, cytoskeletal and chromatin remodelling, and migration.11,12 However, proper Ca2+ handling within the endoplasmic reticulum (ER), the most abundant intracellular Ca2+ reservoir,10 is also crucial for cell-fate decisions. 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The steady-state ER Ca2+ level ([Ca2+]ER) does not only control synthesis, folding, export and trafficking of proteins,10,11 but it also fuels mitochondrial bioenergetics thereby suppressing autophagy.12 Low affinity Ca2+ indicators, such as Mag-Fura 2, and genetically-encoded probes, such as the bioluminescent protein aequorin and the biological fluorochrome aequorin, the green fluorescent protein (GFP) and the red fluorescent protein (mOrange), have been designed to detect and quantify Ca2+ in real-time.13,14 Nevertheless, these sophisticated tools do not provide any reliable information in the solution with Ca2+ more concentrated is NaCl, and 0.5 mM EGTA was added, whereas where Ca2+ has been substituted by 2 mM MgCl2. All the solutions following composition: 150 mM NaCl, 6 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 10 mM Glucose, and 10 mM Hepes; Ca2+-free solution (0 Ca2+), where Ca2+ has been substituted by 2 mM NaCl, and 0.5 mM EGTA was added, whereas the solution with Ca2+ more concentrated is similar to PSS in composition except for 120 mM NaCl and 20 mM CaCl2. All the solutions have been brought to pH 7.4 with an osmolality of 338 mmos/kg, measured with an osmometer (Wescor 5500; Wescor, Inc., Logan, UT, USA).
Intracellular Ca2+ labeling and diaminobenzidine-photoconversion

Intracellular Ca2+ deposits have been marked by fluorescent dye-based calcium indicator Mag-Fura-2, acetoxymethyl (AM) ester UV light-excitable (Life Technologies, Carlsbad, CA, USA), added to the three calcium solutions to obtain a final concentration of 5 μM. Cells have thus been incubated at 37°C in the dark, for 1 h. Cells cultured on chamber slides, and treated as hereto described, have been analyzed at fluorescence microscopy. An Olympus BX51 microscope (Olympus Italia Srl, Segrate, Italy) was used under the following conditions: 100-W Hg lamp as the excitation source, 330- to 385-nm excitation filter (excf), 400-nm dichroic mirror (dm), and 420-nm barrier filter (bf); 100× Olympus UplanFl oil-immersion objective lens (NA 1.25). Images were recorded with an Olympus Magnifier digital camera system, and stored by the Olympus CellF software.

Instead, cells in plates have been fixed at room temperature for 20 minutes with a fixative solution (2% paraformaldehyde and 1% glutaraldehyde in PBS). Meanwhile, a solution with DAB has been freshly prepared as follows: 20 mg of DAB (Sigma Aldrich, St. Louis, MO, USA) were dissolved in 1 ml DMSO, briefly vortexed and then brought to 30 ml with PBS. Thereafter, cells have been washed twice with PBS and incubated for 3 h under a germicidal UV lamp (Sankyo Denki, Kanagawa, Japan; G30T8, UV-C) with 2 mL DAB solution which is renewed and the cells rinsed several times with distilled water, cells have been scraped, centrifuged discarding supernatant, dehydrated in a graded series of ethanol and embedded in LRWhite resin (Sigma Aldrich). Ninety nm ultrathin sections have been cut by a Reichert ultra-microtome, transferred to grids without membrane and visualized by a Zeiss EM900 transmission electron microscope operating at 80kV.

Results

Calcium labeling with Mag-Fura 2 results in highly fluorescent staining mainly localized in the cytoplasm. After incubation of the cells with 20 nM Ca2+ for 1 h, the signal is present in large vesicles in the cytoplasm and around the cell nucleus, as well as in elongated structures likely related to mitochondria (Figure 1a). The faint signal visible in the nucleus is probably due to the superposition of the cytoplasmic layer surrounding it. When the cells are incubated in PSS, the signal is lower (Figure 1b) and mostly present in the perinuclear vesicles, while the cytoplasmic signal far from the nucleus somehow disappears. In the last condition, without calcium and in the presence of EGTA, some labeling is still visible, although to a much reduced level (Figure 1c). This continuous presence of Ca2+ represents very probably the last ions the cell cannot allow to lose, a sort of surviving threshold concentrations. Supplementary Figure 1 confirms that altering extracellular Ca2+ concentrations results in a consequent change in ER Ca2+ homeostasis, which may have important consequences also on cytosolic Ca2+ dynamics.

At EM level, the controls did not show any electron dense precipitates, both in the absence of the fluorochrome or after aldehyde blockade (not shown); consequently, the end product obtained in the Mag-Fura 2 stained cells is due to the fluorochrome photoconversion. The DAB photoconversion is an efficient way to stabilize the ions and to visualize them. It must be underlined here that we observe a thin section, 60-80 nm thick, corresponding, consequently, only to a fraction of the fluorescent signal.

After PSS incubation, the cells show a distinctive positivity (Figure 2a). In some cases it is possible to see the presence of precipitates without the direct association with smooth or rough ER (SER or RER, respectively) membranes, likely related to unbound Ca2+ ions (Figure 2b), and electron dense material inside the vesicles is also positive (Figure 2c). A possible suggestion of Ca2+ internalization or extrusion is shown in Figure 2d,e, where the deposits are present on the outer membrane. When the cells are incubated without calcium and in the presence of EGTA, the deposits are extremely reduced, although still present on the outer cell membrane, suggesting the presence of an extruding mechanism (Figure 2f).

On the contrary, the diffuse, low density end product is almost absent from the cytoplasm. At higher Ca2+ concentration in the medium (Figure 3a), the dark precipitate is detectable on the inner membrane of cytoplasmic vesicles possibly pertaining to SER, and in discrete foci with no apparent association with vesicles or membranes. The deposits are present within electron dense vesicles (Figure 3b) and electron lucent structure, in some cases associated, and suggesting the idea of passage of ions from one compartment to another. In other cases, DAB-positive material is present on the membranes of complex systems such as multivesicular bodies (Figure 3c) or on the outer membranes of cytosolic vesicles (Figure 3d). Interestingly, calcium deposits are detectable on the outer cell membrane (Figure 3e) in spots, localized and separated, suggesting the presence of calcium...
channels. In Figure 3f, another example of surface labelling of calcium ions is presented. The end product of the DAB photoconversion is a speckled, dense precipitate of varying size. However, another abundant end product is present as small sized dots, showing a markedly lower electron density (Figure 3f, inset) and a widespread distribution.

**Discussion**

We show in this paper that Mag-Fura 2 photoconversion via DAB oxidation is an efficient way for localizing Ca\textsuperscript{2+} ions at EM level. Moreover, this could be considered also as a means of stabilizing their presence, thus limiting the loss of ions. As for the method, photoconversion is easily carried out and reproducible, can be obtained on a good amount of cells, since the exposition in our conditions is not limited to the direct irradiation of the sample via an objective but obtained with a germicide lamp. The end product is sufficiently electron dense to be detected clearly when present in sufficient amount within a membrane boundary. In these conditions, the blackness of the product stands out from the background. In our conditions, in fact, Ca\textsuperscript{2+} ions are immediately detectable within the vesicles and the tubules of the SER, while it is much more difficult to see their presence when in the cytosol, due to their low electron density. This is a clear advantage over the conventional imaging of Mag-Fura 2 fluorescence that must be removed by the cytosol in order to study truly ER signals.\textsuperscript{19,20} This is achieved by permeabilizing the plasma membrane with brief applications of antibiotics or detergents, but this procedure may destroy intracellular signaling pathways, dilute cytosolic modulators of ER Ca\textsuperscript{2+} release/sequestration, and disassemble the cytoskeletal elements that maintain the con-
tact between cell membrane and ER. Alternatively, the whole-cell configuration of the patch-clamp technique could be exploited to remove the dye, but this experimental approach is rather expensive and challenging, requires highly skilled electrophysiologists and has a quite low throughput. The final yield of photoconverted Mag-Fura 2 is in direct relation with the amount of Ca present in the sample considered. In our condition, the Ca-enriched medium gave a higher end product staining than the normal physiological conditions or than Ca deprivation.

It is interesting that in several cases, dense precipitates could be found in areas suggesting the local accumulation of Ca\(^{2+}\) nearby the mouth of plasmalemmal Ca\(^{2+}\) channels. Accordingly, such precipitates are evident when extracellular Ca\(^{2+}\) concentration is increased, which is consistent with the notion that constitutive Ca\(^{2+}\) inflow from the extracellular milieu is enhanced. This unexpected outcome of DAB photooxidation is extremely relevant in light of the master role served by local Ca\(^{2+}\) microdomains generated by extracellular Ca\(^{2+}\) influx, which regulate cell functions as diverse as cycle progression, proliferation, migration, nitric oxide production, and adenyl cyclase activity.2\(^{1,2}\) These findings will be further explored in a work now in progress.

One thing that should be underlined here is that we could not visualize the SER tubule filled with the end product. This could be due to three different causes: i) Ca\(^{2+}\) can be dispersed (lost) in the conditions used here; ii) local intraluminal Ca\(^{2+}\) concentration is less than expected (which is less probable as it is unlikely to be lower than 100 \(\mu\)M); or iii) DAB photoconversion is not efficient enough for Mag-Fura. By comparing fluorescence images with EM micrographs, it is clear the difference in the signal coming from vesicles and the elongated filamentous structures, the latter being much less fluorescent.

The relative low yield when comparing fluorescence images is definitely related to the signal coming from vesicles and the elongated filamentous structures, the latter being much less fluorescent. It is of interest that the relative lower amount of end product can be found within a 60-80 nm thin section in relation to the fluorescence originating from the whole cell. In addition, albeit [Ca\(^{2+}\)]\(_{\text{ER}}\) fluctuates between 100 and 500 \(\mu\)M,2\(^{0}\) Ca\(^{2+}\) accumulates in specific regions of the ER\(^{22}\) that may be missed in our sections. Moreover, releasable Ca\(^{2+}\) is actually trapped by oligomers of the Ca\(^{2+}\)-binding proteins calnexin and calreticulin: these form a concealed Ca\(^{2+}\) source that is not in rapid equilibrium with the [Ca\(^{2+}\)]\(_{\text{ER}}\) but is easily accessible to Ca\(^{2+}\)-releasing channels upon extracellular stimulation.2\(^{1}\) Thus, the lack of SER tubules or cisternae filled with the end product of the photo-conversion, i.e., Ca\(^{2+}\)-, is not surprising. Finally, the photoconversion process seems to be efficient, and gives an end product more reasonable than the precipitation technique utilized, for instance, by Tandler et al.\(^{7}\) In the latter case, an important amount of precipitates were found in the cell and especially in the nucleus; our method gives a much finer end product, and this allows a superior resolution and localization.

In conclusion, this method is a reliable and efficient technique for stabilizing and visualizing Ca\(^{2+}\) ions at EM. It gives a high resolution end product thus allowing the fine structural Ca localization. In principle, this approach could provide important qualitative information about the amount and sub-cellular distribution of calcium ions both in cultured cells and, more importantly, in ex vivo samples, which are far less amenable to high resolution Ca\(^{2+}\) imaging of ER Ca\(^{2+}\) levels.

References
1. Hayat H. Stains and cytochemical methods. New York, London, Plenum Press, 1993.
2. Tandler CJ, Libanati CM, Sanchis CA. The intracellular localization of inorganic cations with potassium pyroantimonate. Electron microscope and microprobe analysis. J Cell Biol 1970;45:355-66.
3. Boutinard Rouelle-Rossier V, Biggiogera M, Fakan S. Ultrastructural detection of calcium and magnesium in the chromatoid body of mouse spermatids by electron spectroscopic imaging and electron energy loss spectroscopy. J Histochem Cytochem 1993;41:1155-62.
4. Tsien RY, Pozzan T, Rink TJ. T-cell mitogen causes early changes in cytoplasmic free Ca\(^{2+}\) and membrane potential in lymphocytes. Nature 1982;295:68-71.
5. Bootman MD, Riedtorf K, Collins T, Walker S, Sanderson M. Ca\(^{2+}\)-sensitive fluorescent dyes and intracellular Ca\(^{2+}\) imaging. Cold Spring Harb Protoc 2013;2013:83-99.
6. Dragoni S, Laforenza U, Bonetti E, Lodola F, Bottino C, Berra-Romani R, et al. Vascular endothelial growth factor regulates P2Y12 expression in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2007;27:2360-7.
7. Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 2003;4:517-29.
8. Clapham DE. Calcium signaling. Cell 2007;131:1047-58.
9. Moccia F, Tanzi F, Munaron L. Endothelial remodelling and intracellular calcium machinery. Curr Mol Med. 2014;14:457-80.
10. Sammels E, Parys JB, Missiaen L, De Smedt H, Bulynck G. Intracellular Ca\(^{2+}\) storage in health and disease: a dynamic equilibrium. Cell Calcium 2010;47:297-314.
11. Kiviluoto S, Vereliet T, Ivanova H, Decuyper E, De Smedt H, Missiaen L, et al. Regulation of inositol 1,4,5-trisphosphate receptors during endoplasmic reticulum stress. Biochim Biophys Acta 2013;1833:1612-24.
12. Cárdenas C, Foskett JK. Mitochondrial Ca\(^{2+}\) (Ca\(^{2+}\)) signals in autophagy. Cell Calcium 2012;52:44-51.
13. Demaurex N, Frieden M. Measurements of the free luminal ER Ca\(^{2+}\) concentration with targeted “cameleon” fluorescent proteins. Cell Calcium 2003;34:109-19.
14. Ronco V, Potenza DM, Dentì F, Vello S, Gagliano G, Tognoloni ML, et al. A novel Ca\(^{2+}\)-mediated cross-talk between endoplasmic reticulum and acidic organelles: implications for NADP-dependent Ca\(^{2+}\) signalling. Cell Calcium 2015;57:89-100.
15. Maranto AR. Neuronal mapping: a photooxidation reaction makes Lucifer yellow useful for electron microscopy. Science 1982;217:953-5.
16. Sandell JH, Masland RH. Photoconversion of some fluorescent markers to a diaminobenzidine product. J Histochem Cytochem 1988;36:555-9.
17. Malatesta M, Giagnacovo M, Costanzo M, Conti B, Genta I, Dorati R, et al. Diaminobenzidine photoconversion is a suitable tool for tracking the intracellular location of fluorescently labelled nanoparticles at transmission electron microscopy. Eur J Histochem 2012;56:e20.
18. Malatesta M, Pellicciari C, Cisterna B, Costanzo M, Galimberti V, Biggiogera M, et al. Tracing nanoparticles and photosensitizing molecules at transmission electron microscopy by diaminobenzidine photo-oxidation. Micron 2014;59:44-5.
19. Gerasimenko O, Tepikin A. How to measure Ca\(^{2+}\) in cellular organelles? Cell Calcium 2005;38:201-11.
20. Solovyova N, Verkhovsky A. Measurement of free Ca\(^{2+}\) concentration in the lumen of neuronal endoplasmic reticulum. Cold Spring Harb Protoc 2010;2010: pdb. prot4783.
21. Moccia F, Dragoni S, Lodola F, Bonetti E,
Bottino C, Guerra G, et al. Store-dependent Ca(2+) entry in endothelial progenitor cells as a perspective tool to enhance cell-based therapy and adverse tumour vascularization. Curr Med Chem 2012;19:5802-18.

22. Rizzuto R, Pozzan T. Microdomains of intracellular Ca2+: molecular determinants and functional consequences. Physiol Rev 2006;86:369-408.

23. Guerrero-Hernandez A, Dagnino-Acosta A, Verkhratsky A. An intelligent sarcoplasmic reticulum Ca2+ store: release and leak channels have differential access to a concealed Ca2+ pool. Cell Calcium 2010;48:143-9.