Focal calcium monitoring with targeted nanosensors at the cytosolic side of endoplasmic reticulum

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ABSTRACT

Ca²⁺ distribution is spatially and temporally non-uniform inside cells due to cellular compartmentalization. However, Ca²⁺ sensing with small organic dyes, such as fura-2 and fluo-4, has been practically applied at a single cell level where the averaged signal from freely diffusing dye molecules is acquired. In this study, we aimed to target azide-functionalized fura-2 (N₃-fura-2) to a specific site of subcellular compartments to realize focal Ca²⁺ sensing. Using scAVD (single-chain avidin)–biotin interaction and a copper-free click reaction system, we linked N₃-fura-2 to specifically-targeted scAVD protein fused with a red fluorescent protein mCherry, so that Ca²⁺ sensors conjugated with four N₃-fura-2 dyes with dibenzocyclooctyne (DBCO)-PEG4-biotin as a linker were generated at subcellular compartments in living cells. In cytoplasm, N₃-fura-2 showed a prolonged retention period after binding to scAVD. Furthermore, the reacted N₃-fura-2 was retained inside cells even after free dyes were washed out by methanol fixation. When scAVD was overexpressed on endoplasmic reticulum (ER) membranes, N₃-fura-2 was accumulated on ER membranes. Upon histamine stimulation, which increases cytosolic Ca²⁺ concentration, ER-localized N₃-fura-2 successfully sensed the Ca²⁺ level changes at the cytosolic side of ER membrane. Our study demonstrated specific targeting of N₃-fura-2 to subcellular compartments and the ability of sensing focal Ca²⁺ level changes with the specifically targeted Ca²⁺ sensors.

1. Introduction

Ca²⁺ signaling is crucial in maintaining cellular activities.[1] Due to cellular compartmentalization, Ca²⁺ is non-uniformly distributed between different organelles. Cellular organelles, such as endoplasmic reticulum (ER), mitochondria and Golgi complex, store Ca²⁺ and play fundamental roles in regulating Ca²⁺ levels through Ca²⁺ pumps and release channels on organelle membranes.[2] Monitoring the Ca²⁺ levels in those subcellular organelles as well as in the cytosolic regions that are in close vicinity to those organelles would provide direct evidence in investigating Ca²⁺ signaling pathways and all Ca²⁺-regulated cellular activities.
Calcium imaging at specific subcellular organelles has been generally performed by genetically encoded Ca\textsuperscript{2+} indicators.[3] Compared with those Ca\textsuperscript{2+} indicators, chemical Ca\textsuperscript{2+} indicators have a broader range of Ca\textsuperscript{2+} affinity with current commercially available repertoires.[4] Considering the non-uniformity of Ca\textsuperscript{2+} distribution inside cells, chemical Ca\textsuperscript{2+} indicators may have a wider application (e.g. to detect high Ca\textsuperscript{2+} concentration with a low affinity indicator and to detect low Ca\textsuperscript{2+} concentration with a high affinity indicator [4]). However, like most traditional organic dyes,[5] chemical Ca\textsuperscript{2+} indicators suffer from poor site-specificity and cannot be easily controlled to a particular organelle.[4]

Specific and stable targeting of organic dyes can be realized by tagging organic dyes to proteins. The approaches include various tag-mediated protein labeling,[6,7] site-specific incorporation of unnatural amino acids,[8] and ligand-directed chemistry.[9] Using the tag-mediated labeling technology, several chemical Ca\textsuperscript{2+} indicators have been targeted to nucleus and cytosol in living cells.[10,11] Recently, we have reported a novel three-step method to specifically target organic dyes to intracellular organelles using scAVD–biotin interaction and bioorthogonal reaction between dibenzocyclooctyne (DBCO) and azide, so-called copper-free click reaction (Figure 1).[12] The first step of this method is to overexpress scAVD in living cells. As an advantage of the overexpression system, scAVD can target intracellular organelles by linking to an organelle-targeting sequence. As a tetravalent protein, scAVD is able to bind to four biotin molecules with high affinity.[13] This high affinity enabled efficient attachment of four DBCO-functionalized biotins in the second step. In the third step, azide-functionalized dyes reacted with DBCO by copper-free click reaction, creating a fluorescent probe conjugated with four fluorescent dyes (Figure 1). The probe exhibited increased brightness and enhanced photostability compared with single fluorescent dyes.[12] Therefore, compared with the tag-mediated labeling technology which usually tags one fluorescent dye to a protein molecule, our method could generate probes with a higher signal to noise ratio, which is indispensable for single-molecule imaging. Using this method, we have targeted N\textsubscript{3}-Cy5 and N\textsubscript{3}-TAMRA dyes to the cytosolic side of ER where they tracked ER remodeling with single probe sensitivity and sensed ER temperature changes, respectively.[12]

In this study, we utilized this system to realize stable and specific targeting of N\textsubscript{3}-fura-2,[14] a ratiometric Ca\textsuperscript{2+} indicator, and to generate fluorescent Ca\textsuperscript{2+} sensors conjugated with four N\textsubscript{3}-fura-2 dyes. N\textsubscript{3}-fura-2 has a Ca\textsuperscript{2+} affinity of 304 nM (pH 7.2, 28°C) and a wide sensitivity range (~17 nM to ~39.8 μM).[14] Conjugation to macromolecules by click reaction does not significantly change its Ca\textsuperscript{2+} affinity.[14] The Ca\textsuperscript{2+} sensors conjugated with N\textsubscript{3}-fura-2 were targeted to both cytoplasm and ER membranes at the cytosolic side, for sensing focal Ca\textsuperscript{2+} concentration changes in response to stimulation (Figure 1).
2. Experimental details

2.1. Cell culture

Cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 units ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37°C in the presence of 5% CO₂. All the components were purchased from Invitrogen (Carlsbad, CA, USA). Cells were cultured in 3.5 cm glass-based dishes (IWAKI, Tokyo, Japan) at 37°C with 5% CO₂ for microscopy experiments.

2.2. Plasmids

scAVD DNA was synthesized by Integrated DNA Technologies (Coralville, IO, USA). mCherry-scAVD was generated by insertion of scAVD into a pmCherry-C3 vector. mSTIM1∆2-mCherry and mSTIM1∆2-mCherry-scAVD were generated by insertion of DNA fragment of mSTIM1∆2 (1–343 amino acids) amplified by polymerase chain reaction (PCR) from mouse brain cDNA into the pmCherry-C3 vector and the pmCherry-scAVD-C3 vector, respectively.

2.3. Materials

DBCO-PEG4-Biotin was purchased from Click Chemistry Tools (Scottsdale, AZ, USA). Histamine was purchased form Sigma-Aldrich (St Louis, MO, USA). N₃-fura-2 AM containing an azide moiety and an acetoxymethyl (AM) ester were synthesized according to the procedures previously reported.[14] AM ester will be cleaved by cellular esterase after N₃-fura-2 AM dyes enter cells. Therefore, N₃-fura-2, instead of N₃-fura-2 AM, was used when describing intracellular phenomena.

2.4. Epifluorescence microscopy

Cells were incubated with 10 µM DBCO-PEG4-biotin at 37°C for 1 h followed by incubation with 4 µM N₃-fura-2 AM at 37°C for another 1 h in serum-free DMEM with 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) (Invitrogen). Cells were observed after three washes with phosphate buffered saline (PBS). When fixed cells were observed, cells were treated with 100% ice-cold methanol for 10 min at −20°C. Images were acquired following intracellular phenomena.

2.5. Data analysis

Fluorescence images were analyzed with ImageJ software (National Institute of Health, Bethesda, MD, USA). In Figure 2(b), to calculate the relative intensity, the average fluorescence intensity of F387 of a whole cell at each time point was acquired and normalized to that of the first time point. In Figure 4(b), the average fluorescence intensity of F340 and F387 of a whole cell that over-expressed ER-targeting scAVD was acquired following histamine treatment. The ratio of F340 to F387 was then calculated to show the response profile to histamine. In Figure 4(b), images were shown after photobleaching correction with a CorrectBleach plugin [15]. The p-value shown in Figure 2(c) was calculated by Student's t-test.

3. Results and discussion

Fluorescence images were acquired with ImageJ software (National Institute of Health, Bethesda, MD, USA). In Figure 2(b), to calculate the relative intensity, the average fluorescence intensity of F387 of a whole cell at each time point was acquired and normalized to that of the first time point. In Figure 4(b), the average fluorescence intensity of F340 and F387 of a whole cell that over-expressed ER-targeting scAVD was acquired following histamine treatment. The ratio of F340 to F387 was then calculated to show the response profile to histamine. In Figure 4(b), images were shown after photobleaching correction with a CorrectBleach plugin [15]. The p-value shown in Figure 2(c) was calculated by Student's t-test.
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a plasmid that targets scAVD to ER membranes at the cytosolic side. After incubation with N_3-fura-2 AM, cells were washed with PBS and observed with a microscope. In vector cells, some bright N_3-fura-2 aggregates were observed in the region close to nucleus, probably endocytosed N_3-fura-2. In the remaining regions of the cytoplasm, a very low but evenly distributed N_3-fura-2 signal was present, without accumulation on ER (Figure 3(a)). In contrast to vector cells, although bright N_3-fura-2 aggregates were also present near the nucleus in scAVD-overexpressing cells, N_3-fura-2 showed evident accumulation on ER in the remaining regions of the cytoplasm (Figure 3(a)), presented by a colocalization with ER-targeting scAVD. These results indicate the ER-localization of N_3-fura-2 is due to the binding to scAVD. Fixation with methanol, which dissolves lipids not only on the plasma membrane but also on free dyes existing in the cytoplasm should be washed out. Indeed, scAVD-positive cells (Figure 2(d), cells encircled with red dashed lines) showed a much higher N_3-fura-2 signal than scAVD-negative cells (Figure 2(d), cells encircled with green dashed lines) in which N_3-fura-2 signal is hardly observable. This result further corroborates the notion that N_3-fura-2 successfully bound to scAVD inside living cells by scAVD–biotin interaction and click reaction.

In order to target N_3-fura-2 onto ER membranes at the cytosolic side, HeLa cells were transfected with an ER-targeting scAVD plasmid which contains a truncated mouse STIM1 fragment (mSTIM1Δ2). The mSTIM1Δ2 is an ER transmembrane protein with an N-terminus containing ER-targeting signal at ER lumen and a C-terminus at the cytosolic side.[12,17] The scAVD was fused to the C-terminus of mSTIM1Δ2, creating a plasmid that targets scAVD to ER membranes at the cytosolic side. After incubation with N_3-fura-2 AM, cells were washed with PBS and observed with a microscope. In vector cells, some bright N_3-fura-2 aggregates were observed in the region close to nucleus, probably endocytosed N_3-fura-2. In the remaining regions of the cytoplasm, a very low but evenly distributed N_3-fura-2 signal was present, without accumulation on ER (Figure 3(a)). In contrast to vector cells, although bright N_3-fura-2 aggregates were also present near the nucleus in scAVD-overexpressing cells, N_3-fura-2 showed evident accumulation on ER in the remaining regions of the cytoplasm (Figure 3(a)), presented by a colocalization with ER-targeting scAVD. These results indicate the ER-localization of N_3-fura-2 is due to the binding to scAVD. Fixation with methanol, which dissolves lipids not only on the plasma membrane but also on
the endosomal membrane, led to a virtually complete removal of N$_3$-fura-2 in vector cells, including both the free dyes in cytoplasm and the bright aggregates in endosomes (Figure 3(b)). By contrast, in scAVD-overexpressing cells, N$_3$-fura-2 still remained on ER while bright aggregates disappeared (Figure 3(b)), suggesting the binding of N$_3$-fura-2 to scAVD is stable.

Upon binding to ER, N$_3$-fura-2 is likely to sense Ca$^{2+}$ level changes in cytosolic regions that are in the close vicinity of ER, therefore monitoring the focal Ca$^{2+}$ concentration rather than the average concentration in the whole cell. In order to test the ability of ER-targeting N$_3$-fura-2 to monitor focal Ca$^{2+}$ concentration changes, cells were subjected to the stimulation of histamine, which can increase cytosolic Ca$^{2+}$ level by mediating extracellular Ca$^{2+}$ influx and ER Ca$^{2+}$ release.[18] A Ca$^{2+}$ level increase can be concluded from an increase in the ratio of F340 to F387, and vice versa.[19] Upon histamine stimulation, a sudden increase in F340/F387 ratio was observed (Figure 4(a)), indicating an increase in Ca$^{2+}$ concentration in the region close to ER was monitored by ER-targeting N$_3$-fura-2. To show Ca$^{2+}$ response with the fine structures of ER, we focused on the fluorescence intensity of F387 which was greater than F340 in resting cells when the same imaging parameters were used in our setup. F387 exhibited a sudden decrease in intensity followed by a gradual recovery in response to histamine stimulation (Figure 4(b)). F387 intensity has a negative correlation with Ca$^{2+}$ concentration.[20] Therefore, the fluorescence intensity changes of F387 corresponded to a sudden increase in Ca$^{2+}$ level followed by a gradual drop, which was consistent with the result indicated by the ratio data (Figure 4(a)).

The response of the Ca$^{2+}$ sensors on ER membranes would provide more precise information in judging the involvement of Ca$^{2+}$ channels on ER membranes in a drug-elicited cytosolic Ca$^{2+}$ increase when compared with the response of cytoplasm-localized Ca$^{2+}$ sensors that detect the average cytosolic Ca$^{2+}$ concentration. Furthermore, the Ca$^{2+}$ sensors on ER membranes could be used for direct visualization of the heterogeneous distribution of these Ca$^{2+}$ channels.[21,22] ER subdomains enriched with Ca$^{2+}$ channels may release more Ca$^{2+}$ and create a higher focal Ca$^{2+}$ concentration than the subdomains with fewer Ca$^{2+}$ channels in response to stimulus, resulting in

Figure 3. Fluorescence images showing colocalization of N$_3$-fura-2 with ER in live (a) and fixed (b) scAVD-overexpressing HeLa cells. Regions in squares are zoomed in and shown in right panels. Scale bars, 10 μm for left panels, and 1 μm for right panels.
heterogeneous responses of Ca\textsuperscript{2+} sensors at different subdomains. It is noteworthy that Ca\textsuperscript{2+} diffuse rapidly in cells and the gradients around channels disappear within a few hundred milliseconds.\cite{20} Therefore, a high temporal resolution is required during image acquisition. And the trade-off between temporal resolution and fluorescence intensity should also be considered when setting up the acquisition parameters to ensure a sufficient signal to noise ratio is achieved.

4. Conclusions

In summary, we reported a method for specific and stable targeting of fluorescent organic dyes N\textsubscript{3}-fura-2 in living cells by scAVD–biotin interaction and copper-free click reaction. Using this method, we realized a prolonged retention period of N\textsubscript{3}-fura-2 in the cytosol of cells that is consistent with the previous report.\cite{14} In addition, we successfully targeted N\textsubscript{3}-fura-2 to the cytosolic side of ER membranes. Thus, our study demonstrated the ability of specific targeting of traditional chemical Ca\textsuperscript{2+} indicators and generating Ca\textsuperscript{2+} sensors at specific subcellular organelles. In response to histamine stimulation, which increases cytosolic Ca\textsuperscript{2+} levels, ER-targeting N\textsubscript{3}-fura-2 demonstrated fluorescence intensity changes. These data showed the proof of concept of sensing focal Ca\textsuperscript{2+} concentration changes with specifically targeted Ca\textsuperscript{2+} sensors. Using azide-functionalized dyes with appropriate Ca\textsuperscript{2+} affinities, our method could generate specifically targeted Ca\textsuperscript{2+} sensors at other subcellular compartments, for investigating the Ca\textsuperscript{2+} signaling in different regions of cells.\cite{23–26} Moreover, specifically targeted functional sensors for detecting other chemical and physiological parameters, such as pH and temperature,\cite{12} could also be generated with respective azide-functionalized dyes for studying the establishment and regulation of those parameters in different organelles.\cite{27–30} Our method is therefore a potential tool to develop a wide range of specifically targeted functional sensors for investigation of various localized cellular activities.
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Disclosure statement

No potential conflict of interest was reported by the authors.

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