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COMMENTARY

The RED light is on! New tools for monitoring Ca^{2+} dynamics in the endoplasmic reticulum and mitochondria

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In this issue of the *Biochemical Journal*, Wu et al. describe the characterization of new low-affinity Ca^{2+} indicators for monitoring Ca^{2+} levels in both the ER (endoplasmic reticulum) and mitochondria. In contrast with other ER Ca^{2+} sensors, these indicators emit in the red part of the spectrum, allowing for their use in combination with GFP-based probes and expanding the genetically encoded tool kit for the measurement of Ca^{2+} inside organelles.

Key words: aequorin, Ca^{2+} measurement, endoplasmic reticulum, fluorescent Ca^{2+} indicator, GCaMP, mitochondrion.

Since the identification of the intense Ca^{2+} connection that occurs between the ER (endoplasmic reticulum) and mitochondria, several researchers began to focus their efforts on developing accurate approaches to measure Ca^{2+} in the two organelles. However, it was not long before they realized that this would not be so easy and that the generation of a Ca^{2+} probe with minimal drawbacks would involve travelling a long and winding road.

Indeed, monitoring Ca^{2+} dynamics is considered extremely challenging, especially for the ER due to the intrinsically dynamic nature of the organelle, the complexity of the ER architecture and the difficulty of targeting sensors into the ER lumen in a specific manner.

The ER is considered the major Ca^{2+} storage unit inside the cell ($[Ca^{2+}]_{ER}$ ranges from 500 μM to 1 mM), with significant heterogeneity in Ca^{2+} levels among its different regions. Upon stimulation with agonists such as histamine or ATP, the ER releases Ca^{2+} through the IP_3R (inositol 1,4,5-trisphosphate receptor), ensuring a large accumulation of Ca^{2+} inside the mitochondrial matrix. Mitochondria can rapidly reach high Ca^{2+} levels (from 0.1 to 100 μM in some cellular settings) due to their juxtaposition with ER membranes, the presence of a Ca^{2+}-selective channel and their negatively charged interior [1]. Thus, to operate at these high Ca^{2+} levels, one of the main features an ideal probe must possess for monitoring ER–mitochondria Ca^{2+} dynamics is a very low Ca^{2+} affinity.

Two main genetically encoded strategies are currently used to design a sensor for the measurement of the free luminal ER Ca^{2+} concentration, based on either the bioluminescent protein aequorin or GFP. The use of the photoprotein aequorin provides important benefits, such as highly specific ER staining, a wide dynamic range and a high signal-to-noise ratio, but also some pitfalls, such as low light emission by the photoprotein and its irreversible consumption by Ca^{2+} (for a methodological review on the topic, see [2]). On the other hand, a GFP-based approach allows for the combination of bright fluorescence with good ER targeting. Indeed, the lack of specific localization is the major limitation of all synthetic fluorescent dyes used for measuring Ca^{2+} oscillations in high $[Ca^{2+}]$ compartments. For example, Fluo-5N and Mag-Fluo-4 combine low Ca^{2+}-binding affinities (K_d values for Ca^{2+} of 90 and 22 μM respectively) with easy cell loading, but a majority of the indicator fails to enter the ER and the excessive cytosolic dye must then be removed through 'invasive' methods (patch-clamp or plasma membrane permeabilization), which partially limits the application of these probes [3]. Therefore fluorescent-protein-based Ca^{2+} sensors with genetically encoded chromophores constitute the most commonly used operating system for monitoring Ca^{2+} in the ER.

Historically, ER-targeted cameleon (also called D1ER) represents the most robust and reliable sensor to directly monitor $[Ca^{2+}]_{ER}$ [4]. This indicator is based on FRET and consists of two fluorescent proteins (ECFP and the Venus variant of YFP) linked by the calmodulin-binding peptide M13 and calmodulin. Upon Ca^{2+}-binding, calmodulin wraps itself around the M13 substrate, leading to a conformational change and consequent variations in FRET efficiency.

Previously, two new fluorescent sensors have come to light, confirming that Ca^{2+} measurement inside the ER lumen remains an unexplored territory. CatchER [5] and erGAP1 [6] are both based on GFP, but the first consists of a single GFP molecule engineered by introducing charged residues to bind Ca^{2+}, whereas the latter results from the fusion of GFP and aequorin. All three indicators display some advantages (D1ER and erGAP1 are ratiometric, CatchER possesses an intrinsically low Ca^{2+}-binding affinity) together with potential pitfalls (D1ER has a small dynamic range, CatchER is non-ratiometric and erGAP1 has a relatively high Ca^{2+} affinity), but all of them share one main general feature: they require blue light for excitation and emit in the green part of the spectrum.

Thus the novel Ca^{2+}-indicator described by Wu et al. [7] in this issue of the *Biochemical Journal* is the black (red, in this case) sheep in the (green) flock of ER Ca^{2+} sensors. As a starter template, they used the single RFP-based Ca^{2+} sensor R-GECO1 (red fluorescent genetically encoded Ca^{2+}

Abbreviations: ER, endoplasmic reticulum; LAR-GECO, low-affinity red fluorescent genetically encoded Ca^{2+} indicator for optical imaging; R-GECO, red fluorescent genetically encoded Ca^{2+} indicator for optical imaging.

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indicator for optical imaging 1), as described previously [8]. R-GECO1 belongs to the GCaMP class of genetically encoded Ca^{2+} indicators and consists of a circularly permuted GFP fused to the M13 domain at its N-terminus and calmodulin at its C-terminus. Similarly to the cameleon-like probes, binding to Ca^{2+} causes a structural reorganization of the whole chimaera that leads to an increase in fluorescence. Importantly, R-GECO1 displays a K_d of 0.48 μM. Thus the first step to construct a suitable ER Ca^{2+} probe was to decrease the Ca^{2+} affinity of R-GECO1. The strategic plan, built around a system of random mutagenesis linked to a computationally driven mutagenic approach, allowed the authors to pinpoint the molecular variant expressing the lowest Ca^{2+} sensitivity, while retaining other important features. The so-called LAR-GECO1 (low-affinity red fluorescent genetically encoded Ca^{2+} indicator for optical imaging 1) exhibits a K_d of 24 μM and a 10-fold increase in fluorescence intensity upon binding to Ca^{2+} (dynamic range). Moreover, the computationally based redesign of the calmodulin–M13 interface was implemented to strongly destabilize unwanted interactions with binding factors inside the ER, a previously tested strategy that has been planned and successfully realized to engineer FRET-based Ca^{2+} probes [9].

After the restyling, LAR-GECO1 was ready to be targeted to the ER, validated and compared with other ER Ca^{2+} indicators. The addition of ER-targeting and ER-retention (KDEL domain) sequences allowed for the specific localization of LAR-GECO1 to the ER in different cell types.

Upon the addition of histamine to evoke Ca^{2+} discharge from the ER, ER-LAR-GECO1 displayed a greater decrease in fluorescence compared with CatchER and a higher sensitivity than erGAP1. Furthermore, comparison with D1ER showed similar results in monitoring ER Ca^{2+} dynamics. Thus ER-LAR-GECO1 seems to perform better than the green Ca^{2+} sensors and to measure Ca^{2+} variations with the same accuracy as the well-described and routinely tested ER-cameleon probe.

Importantly, based on the same approach used to characterize ER-LAR-GECO1, another interesting sensor emerged from the molecular screening with characteristics that make it suitable for measuring mitochondrial Ca^{2+} levels. LAR-GECO1.2 (derived from R-GECO1.2) [10] possesses an intermediate Ca^{2+} affinity (K_d of 12 μM), a dynamic range of $\sim$9 and, of course, emits in the red part of the spectrum. Indeed, mito-LAR-GECO1.2 is able to monitor Ca^{2+} inside mitochondria and can be used in association with green fluorescent dyes such as fura 2 for the simultaneous detection of mitochondrial and cytosolic Ca^{2+} oscillations.

In this regard, the most important benefit of the development of red low-affinity Ca^{2+} indicators is the measurement of mitochondrial and ER Ca^{2+} in combination with GFP-based Ca^{2+} reporters or GFP-tagged proteins. Moreover, red sensors do not require blue light for excitation, avoiding a variety of experimental troubles derived from autofluorescence and phototoxicity.

However, some pitfalls do exist: both ER-LAR-GECO1 and mito-LAR-GECO1.2 are non-ratiometric and therefore have all of the limitations of this type of sensor, such as the dependence on probe expression levels and the consequent increase in experimental variability.

In conclusion, low-affinity RFP-based Ca^{2+} sensors are a welcome addition to the toolkit of genetically encoded Ca^{2+} probes, providing new opportunities to measure mitochondrial and ER Ca^{2+} levels in combination with green fluorescent indicators for multicolour imaging.

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