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New Ca^{2+} Indicator has Freedom to Express

In this issue of *Chemistry & Biology*, Tsien and colleagues [1] describe a new family of highly sensitive genetically encoded calcium biosensors that are not affected by their local environment and can measure calcium concentration over a wide dynamic range.

The application of synthetic calcium-chelating indicator dyes to monitor intracellular calcium concentration has provided great insight into the signaling pathways that give rise to changes in intracellular calcium as well as those that are affected by them [2]. Furthermore, they have opened a window onto the intricate temporal and spatial dynamics of intracellular calcium that occur in response to a given stimulus [3]. Without these tools, many of the fundamental signaling mechanisms required for the normal functioning of our cells and organs, such as the consolidation of memory and regulation of the heart beat, would not have been elucidated [4]. Using different experimental conditions, these dyes can be used to measure calcium in intracellular organelles including the ER and mitochondria. However, specific methods usually have to be adopted for each dye and cell type, and the distribution of the dye is not always faithful.

The emergence of genetically encoded calcium indicators that can be specifically targeted to defined intracellular addresses [5] has overcome these problems and has increased significantly our knowledge of calcium dynamics within these domains. The first in situ measurements of intraorganellar calcium levels were obtained using aequorin [6, 7]. Indeed, the first quantitation of calcium transients in living cells was achieved using aequorin purified from photocytes found within the umbrella of the jellyfish *Aequoria victoria*, which had been microinjected into the muscle fibers of giant barnacles [8]. The specialist equipment required to capture the low amount of light emitted by aequorin, and the inability to image its bioluminescence in subcellular domains, has, however, proved an impediment to its widespread use.

The development of green fluorescent protein (GFP)-based calcium indicators has enabled the subcellular imaging of calcium dynamics [9-11]. The first generation of these indicators (known as cameleons) utilized the calcium sensitive binding of calmodulin (CaM) to the M13 peptide (26 aa peptide of myosin light chain kinase) to bring into close proximity two spectral variants of GFP, either a cyan (CFP)-yellow (YFP) or a blue (BFP)-green pair. As a result, radiationless energy transfer (fluorescence resonance energy transfer [FRET]) would occur between the two fluorescent proteins. This is manifest as a decrease in the fluorescence emission of the donor molecule and an increase in the fluorescence emission of the FRET acceptor protein (thus increasing the emission ratio of the indicator) [10]. Tuning of the affinity of the EF-hand calcium binding domains of calmodulin by mutagenesis yielded biosensors with varying affinity for calcium, allowing quantitation of the free calcium concentration from 10^{-8} to 10^{-2} M [10]. Low-affinity versions of cameleon have thus been used to measure free calcium within the lumen of the ER [10, 12]. The low dynamic range (1.1–1.7; the difference between the emission ratio of the calcium bound and calcium free forms of the indicator [13]) of the signal of cameleons has, however, limited their application [14].

The emergence of calcium indicators based on circularly permutated forms of GFP generated increased interest in the use of genetically encoded biosensors to monitor intracellular calcium changes [11, 15]. The generation of these modified fluorescent proteins was made possible through the findings of a random mutagenesis screen, which demonstrated that YFP could fold in two autonomous domains on either side of Y145 [11]. Using this information, Tsien and colleagues replaced Y145 with CaM, generating a calcium-sensitive YFP (known as camgaroo) that exhibited a 7- to 8-fold increase in fluorescence following calcium binding. In a study from Miyawaki's laboratory, YFP was circularly permutated around Y145, with this amino acid forming the new NH_2 terminus. The circularly permutated YFP was rendered calcium-sensitive by appending the M13 peptide to its new NH_2 terminus and calmodulin to its COOH terminus, creating pericams [15]. Calcium-stimulated binding of calmodulin to its M13 target induces a conformational change in the circularly permutated YFP resulting in a change of its intensity of fluorescence and/or spectral characteristics. Although pericams have been successfully targeted to the mitochondrial network and the nucleus [15], to date, one with a sufficiently low affinity for calcium to measure its concentration within the lumen of the ER has not been generated.

The major problems associated with the use of fluorescent protein-based calcium indicators have been: (1) they exhibit sensitivity to pH and chloride, (2) their dynamic range is low, (3) they mature slowly in the harsh environments of intracellular organelles, and (4) they are affected by endogenous levels of CaM, to the extent that in synapses where CaM concentration is high, cameleon indicators are nonfunctional. These issues have been addressed in a number of papers published over the last several years, most recently in a paper by Tsien and colleagues in this issue of *Chemistry & Biology* [1].

YFP is very sensitive to its local environment and in its earlier incarnations did not mature efficiently within the oxidizing environment of the ER. Using a random mutagenesis approach, the laboratories of Tsien and Miyawaki independently obtained variants of YFP, citrine, and venus, respectively, that were relatively pH- and chloride-tolerant and which matured at 37ºC [16, 17]. Replacement of YFP in cameleon with these variants therefore resulted in a significant benefit [18]. To improve the dynamic range of the cameleon indicators, efforts have been focused on minimizing the distance between the FRET partners used in the indicator, thus increasing the FRET efficiency of its calcium bound

form. Ikura and coworkers designed a cameleon containing a Ca^{2+}-sensing module, which relied on the calcium-driven interaction between CaM and CaM-dependent kinase kinase (CKK) [14]. Since this Ca^{2+}-sensing module is shorter than the CaM-M13 peptide, the distance between the donor and acceptor fluorescent proteins in the Ca^{2+} bound state was decreased and the FRET efficiency increased. Replacement of YFP in a cameleon containing the CaM-M13 peptide with a circularly permutated venus molecule, orienting the acceptor in closer proximity with the donor, also had a dramatic effect upon the dynamic range [19]. Substitution of the YFP acceptor in the FRET pair with different fluorophores such as red fluorescent protein from *Discosoma* has also been attempted, although little improvement in the dynamic range was achieved [20].

Griesbeck's group completely reengineered the cameleon indicator. In their sensor, the donor fluorophore was replaced with a circularly permutated improved CFP, cerulean, and the acceptor with a circularly permutated citrine [21]. In addition, they used a modified form of the calcium sensor from skeletal and cardiac muscle Troponin C instead of CaM. As well as exhibiting a very rapid response time and off rate, and a 400% increase in fluorescence upon calcium binding, this indicator (unlike CaM-M13 peptide-containing indicators) was not affected by nor did it affect endogenous proteins.

In the manuscript by Palmer et al. [1], a rational approach was adopted to design an indicator that exhibited a large dynamic range and that did not interact with endogenous CaM. In this way, the indicator could measure very small changes in calcium concentration in cellular microdomains that contained high levels of CaM. Taking the crystal structure of CaM bound to smooth muscle myosin light-chain kinase as a starting point, they used a computational algorithm to predict the effects of performing an alanine scan upon the protein. The replacements that resulted in the greatest change in binding energy were identified as hot spots. The authors then replaced small amino acids within these hot spots with bulkier hydrophobic residues that would perturb the interaction with endogenous CaM. A second round of analysis was then carried out to determine the changes in wild-type CaM required to optimally bind to the modified peptide. Selection of peptide pairs for further analysis was based on whether they exhibited an equal or higher affinity for each other than did the wild-type pairs and whether the modified peptide had a low affinity for wild-type CaM. The indicators with the best dynamic range (5- to 6-fold change in emission ratio upon calcium binding) were generated by placing the peptide pair between CFP and a circularly permutated venus. By tuning the affinity of the CaM/peptide interaction and using the EF-hand mutations in CaM utilized in previous incarnations of cameleons, these new indicators report Ca^{2+} concentration over a continuous very wide spectrum, from the nanomolar levels observed in the cytosol to the high micro- and millimolar concentrations at the mouth of plasma membrane-localized channels. In addition, they are not affected by local environment and can be successfully targeted to the mitochondria and plasma membrane.

The Troponin C-based indicator [21] may have an advantage over the indicator described by Palmer et al. [1], in that it responds more rapidly to changes in calcium. However, the new cameleon may prove more robust, as its completely engineered sensor interactions may not be subject to interference from other cellular proteins in a way that the calcium sensor in the Troponin C-based indicator might.

There is no doubt that genetically encoded calcium indicators will continue to improve and will enable the measurement of calcium in all subcellular domains and in the organs of transgenic animals. Moreover, the methods used in this paper by Tsien and colleagues [1] may find broad application to design better indicators for other cellular signaling molecules and metabolites.

H. Llewelyn Roderick[1,2] and Martin D. Bootman[2]
[1] Department of Pharmacology
University of Cambridge
Tennis Court Road
Cambridge, CB2 1PD
United Kingdom
[2] Laboratory of Molecular Signalling
Babraham Institute
Cambridge CB2 4AT
United Kingdom

Selected Reading

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