Point of VIEW

ACS chemical biology

Expanding the Repertoire of Fluorescent Calcium Sensors

Amy E. Palmer*

Department of Chemistry and Biochemistry, University of Colorado, UCB 215, Boulder, Colorado 80309

ABSTRACT Fluorescent indicators for calcium are incredibly powerful because they enable researchers to watch the movement of calcium ions in real time in living cells. The popular small molecule indicator Indo-1 has now been targeted to a defined location, namely, the nucleus of muscle cells, using SNAP-tag technology. This combination of a chemical probe with genetic targeting expands the available options for measuring local calcium events in cells.

The calcium ion (Ca^{2+}) comprises a fundamental cellular signal that controls critical processes such as cell growth, proliferation, gene regulation, and cell death. Although calcium is essential, it is highly toxic, and therefore cells must tightly regulate the nature of Ca^{2+} signals. These signals are defined as transient increases in Ca^{2+} over basal resting levels, and they vary in amplitude, frequency, and spatial localization. It is by precisely regulating both the spatial and temporal aspects of Ca^{2+} signals that cells have developed the means to control a host of diverse processes. Fluorescent indicators for Ca^{2+}, coupled with live cell imaging, provide the exceptional ability to watch Ca^{2+} signals in real time, thus enabling researchers to define the vast array of Ca^{2+} signals and how these signals regulate cellular processes. In this issue of *ACS Chemical Biology*, Bannwarth *et al.* (DOI 10.1021/cb800258g) (*1*) combine the versatility of small molecule probes with the power of genetic targeting by coupling the fluorescent Ca^{2+} indicator Indo-1 to a SNAP-tag. The SNAP-tag technology permits targeting of a Ca^{2+} indicator to a specific location within a cell. Such a localized probe is essential for monitoring Ca^{2+} in cellular organelles such as the endoplasmic reticulum or mitochondria and in microdomains such as at the mouth of calcium channels.

The distribution of Ca^{2+} in cells is highly heterogeneous (Figure 1). Resting cells maintain cytoplasmic Ca^{2+} at ~100 nM, and there is little fluctuation in this value as a result of the prevalence of cytoplasmic buffers and feedback mechanisms to maintain this level, which is 10^4 times lower than the extracellular milieu. The endoplasmic reticulum serves as a storehouse of Ca^{2+}, containing hundreds of μM. Because Ca^{2+} is toxic, cells have evolved exquisite mechanisms for regulating its availability. Ca^{2+} signals can be generated by release of Ca^{2+} from the endoplasmic reticulum or by influx across the plasma membrane. These signals are modulated by proteins, channels, and pumps that move elevated Ca^{2+} outside of the cell, enable re-uptake into the endoplasmic reticulum, and facilitate translocation into mitochondria.

Over 20 years ago Tsien and co-workers (*2*) developed a series of small molecule probes to visualize the movement of calcium in cells. Binding of Ca^{2+} to the probes causes either a change in fluorescence intensity or a spectral shift, thus enabling researchers to track Ca^{2+} dynamics in live cells using fluorescence microscopy. Since that time, a vast repertoire of fluorescent Ca^{2+} indicators has been developed. These probes generally fall into three classes: (i) small molecule fluorescent indicators; (ii) fluorescent calcium indicator proteins (FCIPs), also sometimes referred to as genetically encoded calcium indicators (GECIs); and (iii) chemiluminescent Ca^{2+} sensors based on the protein aequorin. Such probes have had a profound impact on our understanding of how cells control the nature of Ca^{2+} signals and in turn how Ca^{2+} signals direct different cellular pro-

***Corresponding author,**
amy.palmer@colorado.edu.

Published online March 20, 2009
10.1021/cb9000525 CCC: $40.75

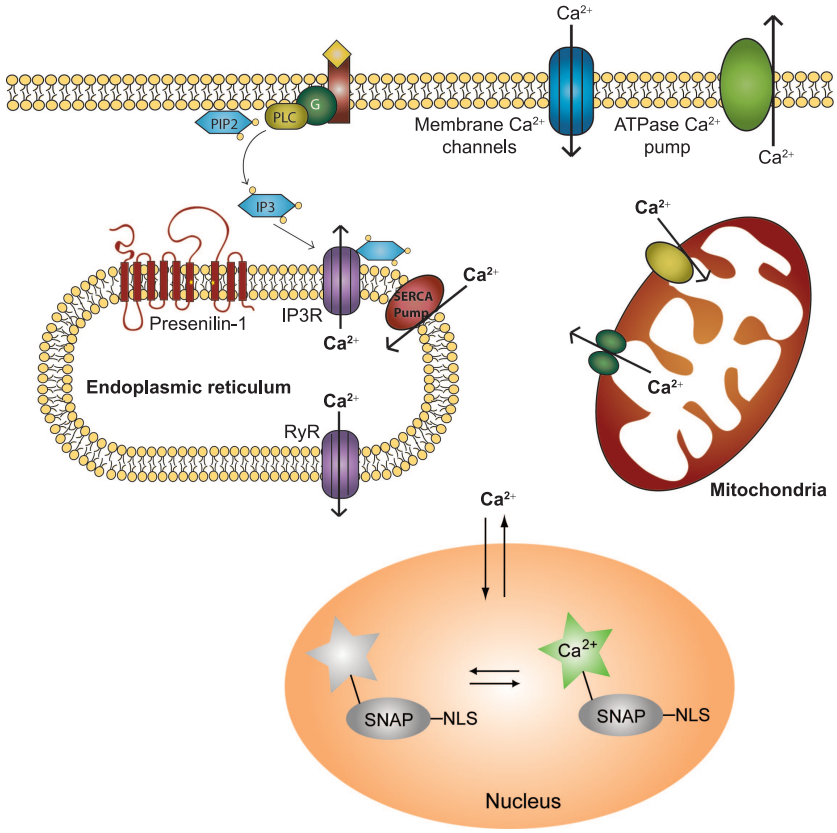

Figure 1. Schematic of the spatial distribution of cellular Ca^{2+} and the application of SNAP-Indo-1 to examine localized Ca^{2+} signals. SNAP-Indo-1 is shown localized to the nucleus using a nuclear localization signal. Ca^{2+} binding leads to a change in the fluorescence signal. Although Ca^{2+} is distributed in many different locations in the cell, the localized probe ensures that only nuclear Ca^{2+} signals give rise to a fluorescence change. Potential exciting future directions include localization of Ca^{2+} indicators to the endoplasmic reticulum, mitochondria, and channels on the plasma membrane. Abbreviations: NLS, nuclear localization signal; IP3R, inositol 1,4,5-triphosphate receptor; RyR, ryanodine receptor, SERCA, sarco-endoplasmic reticulum ATPase; IP3, inositol 1,3,5-triphosphate; PIP2, phosphotidylinositol-4,5-bisphosphate; PLC, phosolipase C. Figure credit: Janet McCombs.

cesses. For example, oscillations in Ca^{2+} lead to specific transcriptional responses, and the frequency (rather than the amplitude) of oscillations is "decoded" by effector proteins (*3–5*). Additionally, translocation of Ca^{2+} from one location (the endoplasmic reticulum) to another (the mitochondria) controls one pathway of programmed cell death. Such mechanistic detail would not have been possible without the development of fluorescent probes that enabled real time imaging of Ca^{2+} in cells.

Localized probes are incredibly valuable because they enable dissection of the origin and destination of Ca^{2+} signals. For example, to measure Ca^{2+} dynamics within mitochondria, researchers require an indicator that can be explicitly targeted to mitochondria with no interference from Ca^{2+} signals in the cytoplasm. Historically it has been difficult to direct small molecule probes to specific places in the cell. Thus, to accomplish targeting researchers turned to genetically encoded sensors (FCIPs/GECIs or aequorin-based probes). These fluorescent sensors are generated by translation of a nucleic acid sequence and are incorporated into cells by transfection or transgenic technologies. They can be targeted to specific locations within a cell by fusion to a protein of interest or incorporation of signal sequences, where signal sequences act like zip codes and send the sensor to the desired location. Through continual optimization, FCIPs have made considerable strides in monitoring local Ca^{2+} signals in the endoplasmic reticulum and mitochondria and at the plasma membrane (*6–10*). Despite these gains, when compared to small molecule fluorescent indicators, the FCIPs exhibit a lower dynamic range and slower response kinetics. In 2007, Tsien and coworkers (*11*) developed a hybrid sensor comprising the small molecule indicator calcium green FlAsH, which bound a 12-amino-acid genetically targeted tetracysteine tag. This hybrid system enabled the first direct measurements of Ca^{2+} at the mouth of Ca^{2+} channels. Still, a number of characteristics such as the requirement for a reducing environment and the lack of a ratiometric indicator limit its utility to specific situations. To broaden the tools available to researchers, it would be incredibly valuable to be able to target the wealth of small molecule indicators to explicitly defined locations. By coupling the SNAP-tag technology to Indo-1, Bannwarth *et al.* have now established the feasibility of this approach, opening new doors for sensing local Ca^{2+} signals.

The SNAP-tag technology developed by Johnsson and co-workers (*12, 13*) facilitates site-specific covalent labeling of a protein of interest with a chemical probe. To adapt this technology for localization of calcium sensors, the authors synthesized derivatives of benzylguanine linked to Indo-1 (referred to as BG-Indo-1). Indo-1 is a small

molecule fluorescent indicator whose emission spectrum shifts upon Ca^{2+} binding. Indo-1 is widely used in cell biology because the fluorescence change is ratiometric, the dynamic range (i.e., the signal change between the Ca^{2+}-free and Ca^{2+}-bound forms) is large, and the response kinetics are fast. Three different BG-Indo-1 derivatives were synthesized that differed in the linker between Indo-1 and the benzylguanine moiety. In one of these derivatives (BG3-Indo-1) the Ca^{2+}-binding carboxylates were capped by acetomethoxy esters, making the indicator membrane permeable for facile loading into cells. The BG-Indo-1 derivatives reacted readily with the SNAP-tag *in vitro*, yielding protein covalently labeled with Indo-1 (referred to as SNAP-Indo-1). Through *in vitro* experiments Bannwarth *et al.* demonstrate that the binding affinity and the quantum yield of the Ca^{2+}-free and Ca^{2+}-bound forms of the SNAP-Indo-1 derivatives are comparable to that of the free Indo-1 indicator, although the dynamic range decreased from 24× to ~9×.

To demonstrate the new probe's functionality in cells, Bannwarth *et al.* genetically fused the 182-amino-acid SNAP tag to a nuclear localization sequence in order to target the SNAP-tag to the nuclei of mouse muscle fibers (Figure 1). They show that the sensor responds to changes in cellular Ca^{2+} and that the *in situ* K_d is comparable to that measured *in vitro*. This work expands the repertoire of hybrid small molecule−genetic targeting approaches and opens up the possibility of using the vast array of small molecule Ca^{2+} indicators to measure local Ca^{2+} signals. It is important to note that this technology is not limited to Ca^{2+} indicators but should be applicable to genetic targeting of other chemical probes as well. Recently Lippard and colleagues (*14*) generated zinc indicators localized to mitochondria and Golgi using the SNAP-tag technology.

While the present study establishes an exciting new direction for the development of localized indicators, there are still issues that need to be addressed. First, researchers will need to determine how to maintain the large dynamic range of small molecule sensors when the probes are linked to SNAP-tags in cells. It is unfortunate that the dynamic range of the Indo-1 indicator decreases by about half for the SNAP-tagged versions (24× *vs* 9× *in vitro* or 7.8× *vs* 3.8× in cells), particularly because one of the distinct advantages of Indo-1 is its large dynamic range. A dynamic range of 3.8× is comparable to (or slightly less than) that of localized FCIPs. However the study of Bannwarth *et al.* hints that perhaps this issue can be addressed by variation of the benzyl linker, as they observed variation of dynamic range *in vitro* for different derivatives (9× *vs* 15×). Second, it will be important to assess if localized probes retain fast response kinetics. Third, extension of this technology to other organelles would be very useful. The BG-Indo-1 is small enough to diffuse through the nuclear pore, and therefore there is essentially no barrier to nuclear labeling. It may be more challenging for the probe to access the lumen of the endoplasmic reticulum or matrix of mitochondria at high enough levels to achieve adequate signal over background autofluorescence. Still, the recent study by Lippard and colleagues demonstrating SNAP-targeting of a zinc sensor to mitochondria and Golgi suggests that access to these locales is possible. It is likely that these issues will be addressed with optimization and extension of this technology to ever-broader applications. Thus, the work of Bannwarth *et al.* opens a new door for targeting chemical probes to specific localizations in cells.

REFERENCES

1. Bannwarth, M., I. R., Correa, J., Sztretye, M., Pouvreau, S., Fellay, C., Aebischer, A., Royer, L., Rios, E., and Johnsson, K. (2009) Indo-1 derivatives for local calcium sensing, *ACS Chem. Biol. 4*, (3), 179–190.
2. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, *J. Biol. Chem. 260*, (6), 3440–3450.
3. Dolmetsch, R. E., Xu, K., and Lewis, R. S. (1998) Calcium oscillations increase the efficiency and specificity of gene expression, *Nature 392*, (6679), 933–936.
4. Li, W.-h., Llopis, J., Whitney, M., Zlokarnik, G., and Tsien, R. Y. (1998) Cell-permeant caged InsP3 ester shows that Ca^{2+} spike frequency can optimize gene expression, *Nature 392*, (6679), 936–941.
5. De Koninck, P., and Schulman, H. (1998) Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations, *Science 279*, (5348), 227–230.
6. Palmer, A. E., Jin, C., Reed, J. C., and Tsien, R. Y. (2004) Bcl-2-mediated alterations in endoplasmic reticulum Ca^{2+} analyzed with an improved genetically encoded fluorescent sensor, *Proc. Natl. Acad. Sci. 101*, (50), 17404–17409.
7. Filippin, L., Magalhaes, P. J., Di Benedetto, G., Colella, M., and Pozzan, T. (2003) Stable interactions between mitochondria and endoplasmic reticulum allow rapid accumulation of calcium in a subpopulation of mitochondria, *J. Biol. Chem. 278*, (40), 39224–39234.
8. Scorrano, L., Oakes, S. A., Opferman, J. T., Chang, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. (2003) BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis, *Science 300*, (5616), 135–139.
9. Heim, N., Garaschuk, O., Friedrich, M. W., Mank, M., Milos, R. I., Kovalchuk, Y., Konnerth, A., and Griesbeck, O. (2007) Improved calcium imaging in transgenic mice expressing a troponin C-based biosensor, *Nat. Methods 4*, (2), 127–129.
10. Palmer, A. E., Giacomello, M., Kortemme, T., Hires, S. A., Lev-Ram, V., Baker, D., and Tsien, R. Y. (2006) Ca^{2+} indicators based on computationally redesigned calmodulin-peptide pairs, *Chem. Biol. 13*, (5), 521–530.
11. Tour, O., Adams, S. R., Kerr, R. A., Meijer, R. M., Sejnowski, T. J., Tsien, R. W., and Tsien, R. Y. (2007) Calcium Green FlAsH as a genetically targeted small-molecule calcium indicator, *Nat. Chem. Biol. 3*, (7), 423–431.
12. Keppler, A., Gendreizig, S., Gronemeyer, T., Pick, H., Vogel, H., and Johnsson, K. (2003) A general method for the covalent labeling of fusion proteins with small molecules *in vivo*, *Nat. Biotechnol. 21*, (1), 86–89.
13. Gronemeyer, T., Chidley, C., Juillerat, A., Heinis, C., and Johnsson, K. (2006) Directed evolution of O6-alkylguanine-DNA alkyltransferase for applications in protein labeling, *Protein Eng. Des. Sel. 19*, (7), 309–316.
14. Tomat, E., Nolan, E. M., Jaworski, J., and Lippard, S. J. (2008) Organelle-specific zinc detection using zinpyr-labeled fusion proteins in live cells, *J. Am. Chem. Soc. 130*, (47), 15776–15777.