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New and Notable

$Ca^{2+}{}_i$ versus $[Ca^{2+}]_i$

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The importance of intracellular ionized calcium levels ($Ca^{2+}{}_i$) as a second messenger is no longer controversial. A vast literature now documents the involvement of $Ca^{2+}{}_i$ in signal transduction pathways in a myriad of cell types (1). That $Ca^{2+}{}_i$ levels change is not in question. Rather the questions have become: Exactly what is the quantitative value of the $[Ca^{2+}]_i$ and what are the kinetics of changes in $[Ca^{2+}]_i$ in the intact cell? These questions are the topic of the work described in the article by Harkins, Kurebayashi, and Baylor in this issue.

Three major types of techniques are available for monitoring $Ca^{2+}{}_i$ levels: ion-selective electrodes, nuclear magnetic resonance, and optical calcium indicators. The use of ion-selective electrodes has been limited (except for a few brave souls) to large cells and even in these cells the possibility of artifactual increases in $Ca^{2+}{}_i$ caused by impalement of the electrode into the cell is difficult to rule out. The use of symmetrically substituted difluoroderivatives of 1,2-bis(2-aminophenoxy)-ethane N,N,N',N'-tetraacetic acid (BAPTA) as NMR probes has been available since 1983 (2), however the relatively high concentrations of the probe, large tissue sample sizes, and long acquisition times required for these measurements has limited utility of this approach.

Thus, the vast majority of measurements of $[Ca^{2+}]_i$ in the current literature are being made with optical calcium indicators. Again, there are three major classes: the metallochromic dyes, the luminescent calcium indicators, and the fluorescent calcium indicators. The metallochromic indicators (including arsenazo-III and antipyrylazo III) are being little used in the current literature, probably because of the intrinsic difficulties of making the required absorbance measurements and the possibility of artifacts (especially in contractile cells) associated with such measurements. The main luminescent indicator in current use is aequorin, a natural product from jellyfish of the genus *Aequoria*. Its use is restricted to a relatively small group of investigators, primarily because of the lack of commercial availability of the equipment required and initial difficulties involved in introducing this 22-kDa protein into living cells, although several creative techniques appear to have bypassed this problem (3). These are the only protein-based $Ca^{2+}{}_i$ indicators, and the recent reports of transfecting cells in a targeted manner with aequorin cDNA has resulted in renewed interest in this class of indicators (4).

Although the metallochromic and luminescent indicators were in use in the 1960s, the use of calcium indicators did not become widespread until the late 1980s, after the first paper on the fluorescent tetracarboxylate indicator fura-2 was published from Roger Tsien's group (5). The 1985 paper on this topic was closely followed by the commercial availability of both the dye and the equipment necessary for using the dye which resulted in a dramatic expansion in the calcium literature. The main appeal of fura-2 arose from the fact that, upon binding calcium, the dye displays a spectral shift allowing "ratio" measurements which mathematically eliminate a wide range of potential artifacts due to photobleaching, dye loss, and changes in tissue thickness. Another major factor in the popularity of fura-2 was its availability in the cell-permeant acetoxymethylester form. This allowed for passive trapping of fura-2 in the cytosol upon interaction with cellular esterases, thus avoiding the necessity for microinjection or complex loading procedures.

Although a vast literature now exists on the use of tetracarboxylate dyes to monitor $Ca^{2+}{}_i$ in diverse cells, a disproportionately smaller literature has addressed the important question of the accuracy of the available calibration methods. Many investigators continue to use the original in vitro method with a K_d published for an arbitrary calibration solution (5). This is often done without justification and often leads to obviously erroneously low (and in some case negative) estimations of $[Ca^{2+}]_i$. The most common source of error has unfortunately turned out to be partial hydrolysis of the acetoxymethylester groups, leading to an artifactual calcium-insensitive fluorescence in the same range of wavelengths associated with the non-calcium bound form of the tetracarboxylate dyes, thus, giving the appearance of a lower $[Ca^{2+}]_i$ than that really existing.

Ironically, the best solution to avoid partially hydrolyzed forms of these dyes appears to be the return to microinjection, again raising questions concerning damage-induced increases in $[Ca^{2+}]_i$. Even using microinjected tetracarboxylate dyes, however, there still remain major questions as to the validity of calibration methods used. Several studies (1) have clearly shown that the fluorescence properties of these dyes in myoplasm differs from that in artificial calibration solutions. What is not clear is the proper method of correcting for the influence of myoplasm. Calibration solutions have been made with increased viscosity or with the addition of substances to mimic the binding of the indicator molecules to intracellular constituents. The paper by Harkins et al. in this issue addresses the inadequacy of those methods and presents an entirely new method for the calibration of the tetracarboxylate calcium indicator fluo-3. The method utilizes a combination of fluorescence and absorbance measurements to allow a noninvasive quantitation of $[Ca^{2+}]_i$ in the myoplasm of the intact cell. Interestingly, the resulting estimates of resting $[Ca^{2+}]_i$ appear to be somewhat higher than previous estimates using other indicators and other calibration techniques.

Another major limitation of the tetracarboxylate calcium indicators alluded to in the Harkins et al. paper is that

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the time course of the fluorescent signal appears to substantially lag behind that of the myoplasmic $[Ca^{2+}]_i$ transient. This has most clearly been shown in past studies by Baylor and coworkers where fluorescent calcium indicators were coloaded with metallochromic dyes into individual cells. In general, the approach of simultaneously using multiple different methods of $[Ca^{2+}]_i$ measurement is a strong one, and indeed one of the most puzzling aspects of the Harkins et al. paper is that the estimate of $[Ca^{2+}]_i$ is higher than that obtained by the simultaneous use of aequorin and calcium-selective electrodes by Blatter and Blinks. Additional studies combining the diverse methods available for the measurement of $[Ca^{2+}]_i$ will probably be necessary to finally produce measurements of $[Ca^{2+}]_i$ that are beyond debate.

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