# Calcium on the level

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The vast majority of the now over 1,000 papers that have used calcium indicators have employed them to answer qualitative questions. In many cases it was only important to know whether free $[Ca^{2+}]$ went up or down in response to a particular stimulus. For these applications the easily measured fluorescence changes of fura-2 and its analogues have made them the indicators of choice. However, other problems demand more precise answers for which the cookbook ratiometric formula (1) may not be sufficient. What exactly are the $[Ca^{2+}]$ values in a cell where the cytoplasmic environment makes the cuvette calibrations of the indicator response unreliable? What are the true peak values and time course of $[Ca^{2+}]$ transients when these parameters are influenced by the indicators used to measure them?

For these kinds of questions the muscle fiber presents both a challenge and an opportunity. It is a challenge first because action potentials evoke $Ca^{2+}$ transients of larger amplitude and faster time course than found in most other cells. The limited dynamic range and slow rate constants of fura-2 has limited its usefulness in this area. A second challenge is that for problems of current interest it truly matters what the absolute $[Ca^{2+}]$ levels are. Our understanding of the biochemistry of muscle contraction has reached a point where actual numbers and not just estimates are needed to compare *in vivo* and *in vitro* measurements.

The opportunity offered by the muscle preparation is that muscle fibers are large and of relatively uniform shape compared with other cell types. Also, to a first approximation, calcium is released from the sarcoplasmic reticulum in a spatially uniform manner. This is important since optical recording techniques necessarily average measurements over a volume and consequently are inaccurate for following localized $Ca^{2+}$ transients, like, for example, those induced near plasma membrane $Ca^{2+}$ channels in neurons.

Over the past fifteen years many investigators have used a variety of ion-sensitive indicators to try to understand calcium dynamics in muscle fibers. Much effort has gone into an examination of the indicators themselves, since the experiments exposed calibration problems and annoying errors not noticed in the more qualitative experiments on other cell types. (See, for example, the revealing studies of arsenazo III from Chandler's laboratory [2, 3]). The latest attempt to slay these demons is a very careful measurement of resting $[Ca^{2+}]$ using fura red, a fluorescent $Ca^{2+}$ indicator with absorbance bands at visible wavelengths, reported in this issue by Kurebayashi, Harkins, and Baylor (4).

Two parameters must be determined in a measurement of resting $[Ca^{2+}]$. These are $K_D$, the dissociation constant for the $Ca^{2+}$-indicator reaction, and $f_r$, the fraction of indicator molecules bound to $Ca^{2+}$ at rest. The problem is that both of these constants are strongly affected by the intracellular milieu. One factor is that, as in most cells, myoplasmic viscosity is significantly higher than in buffer solutions normally used for fura-2 calibrations. In addition, most indicator molecules (estimated at ~85% in this study) are bound to muscle constituents of high molecular weight. Both of these factors were found to affect the fluorescence properties of an indicator much more than its absorbance properties. Kurebayashi, Harkins, and Baylor took advantage of this fact to calibrate fura red using absorbance spectra. The particular advantage of fura red over the previously studied fura-2 (5, 6) is that its absorbance spectrum lies in the visible region rather than the near UV where fura-2 responds. For technical reasons accurate measurements in the near UV region were difficult. The authors then used simultaneously recorded absorbance and fluorescence transients to construct an "in situ" calibration curve for the indicator's fluorescence properties under physiological conditions. This curve, in turn, was employed to determine resting $[Ca^{2+}]$ at locations away from the injection site where the lower indicator concentrations reduce the consequences of indicator buffering. The effects of electrode penetration damage also are expected to be minimal at these distant locations. Along the way many other potential problems were examined and appropriate corrections were incorporated into the final result. Remaining errors are clearly delineated.

The authors found that the most likely $K_D$ for the $Ca^{2+}$-fura red reaction in a muscle fiber is close to 1 $\mu$M, about three times larger than the cuvette value, a shift that could largely be mimicked by adding the common cytoplasmic protein aldolase to the calibrating buffer. Combining this value with the *in vivo* determined $f_r$ = 0.15, they found resting free $[Ca^{2+}]$ to be in the range 0.18–0.27 $\mu$M, significantly higher than previous estimates in frog muscle fibers made with other calcium indicators or electrodes.

A different way to measure resting $[Ca^{2+}]$ avoids problems with sensor calibration and relies instead on the detection of a null response to solutions of known $[Ca^{2+}]$. This approach was used originally by Baker, Hodgkin, and Ridgeway (7) in their pioneering study of $Ca^{2+}$ transients. Squid giant axons were loaded with the luminescent protein aequorin and then injected with strongly buffered solutions of different calcium concen-


0006-3495/93/06/1655/02 $2.00

trations. Resting [$Ca^{2+}$] was roughly estimated as 0.3 $\mu$M, with the greatest uncertainty attributed to the accuracy of the standards. This method has not been seriously pursued, perhaps because subsequent experiments have shown that the resting aequorin glow is close to zero in healthy cells. However, recent technical improvements have prompted a renewal of this approach. Specially modified or recombinant aequorins are more sensitive at low [$Ca^{2+}$] levels. Ridgeway and Gordon (8) injected these new photoproteins into barnacle muscle fibers and measured the luminescence with a sensitive camera. With this detector they could selectively evaluate the response at sites away from the injection site, avoiding artifacts due to injection damage. Using standard solutions buffered with either EGTA or BAPTA they determined a resting free calcium level of 0.34 $\mu$M in barnacle muscle. Thus, both of these new techniques find higher calcium levels than previously assumed. At this point it would seem important to use both methods on the same preparation to see whether the same value of resting free [$Ca^{2+}$] is obtained.

These new experiments raise the possibility that resting levels of free [$Ca^{2+}$] in other cell types may also be higher than the 50–100 nM levels commonly assumed. Kurebayashi et al. present evidence in support of this idea. They point out that proteins like aldolase which affect the calibration and fluorescence properties of fura-2 and fura red are found in many other cells. Also, their *in situ* fluorescence anisotropy measurements show that these indicators are bound predominantly to soluble proteins and not the oriented fibrillar proteins which are unique to muscle. Some careful measurements in neurons and other cells using these new methods would seem to be in order.

## REFERENCES


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