New and Notable

Calcium Buffers in Flash-Light

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Ca^{2+}-binding proteins are dear to neuroanatomists because antibodies against them selectively stain specific subpopulations of neurons in the brain (Andressen et al., 1993). Thus, one might conclude that they have very specific functions, conferring distinct properties on the cell populations in which they are expressed. Unfortunately, it must be concluded, however, that we hardly understand what the staining pattern tells us, as the functional significance of Ca^{2+} binding proteins is only slowly emerging. Part of the reason for this ignorance is the fact that there is a not so trivial interplay between Ca^{2+} binding proteins, other cellular Ca^{2+} buffers, Ca^{2+} release, and Ca^{2+} extrusion mechanisms, not to mention the Ca^{2+} indicator dyes that are used to measure Ca^{2+} signals. To understand some aspects of this interaction we not only need to know the amounts and affinities of the Ca^{2+} chelators, but also their kinetic properties. The paper by Naegerl et al. (2000) in this issue represents a big step toward providing such basic information by measuring rate constants for Ca^{2+} binding and dissociation of one of the most abundant Ca^{2+}-binding proteins, calbindin-D28k. This is achieved by analyzing the time course of binding of Ca^{2+} to the buffer after uncaging Ca^{2+} with a UV flash. The method should be readily applicable to other Ca^{2+}-binding proteins and should be portable to in vivo experiments.

The action of fast Ca^{2+} buffers (the term Ca^{2+} buffer will be restricted here to proteins and other ligands binding Ca^{2+} and will not be used for other Ca^{2+} sequestration mechanisms) seems to be quite trivial—to bind Ca^{2+} rapidly and thereby lower its concentration. This, however, is true only transiently; for instance, after a short episode of Ca^{2+} influx. As time goes on, Ca^{2+} pumps will remove Ca^{2+} from the cytosol and from the buffers, establishing a steady state that is determined by a balance between Ca^{2+} fluxes across the boundaries surrounding the space occupied by cytosol. The presence of buffers inside will not influence that balance and, therefore, not change steady-state Ca^{2+} levels. Buffers will, however, lengthen the time required for the steady state to be achieved. In simple cases this lengthening will be by exactly the same factor by which the amplitude of the transient is reduced, such that the area under the transient (or the product of amplitude and time constant) will not be influenced by the presence of the buffer. The same will be true for the cumulative effect on any process that is linearly dependent on Ca^{2+} (Neher, 1998). This simple consideration gives a hint as to why it is so difficult to pinpoint the action of fast Ca^{2+} buffers: it resides mainly in deviations from linearity of the Ca^{2+}-effector systems. In order to appreciate this, accurate numbers on affinities are required.

Another issue is kinetics. While a fast buffer will simply diminish the amplitude and slow down a Ca^{2+} transient (unless it becomes saturated), a slow buffer will affect the time course of the Ca^{2+} signal in a more complicated manner: a rapid decay phase, which represents binding of Ca^{2+} to the buffer, will be followed by a return to baseline, which is slower than what would occur in the absence of buffer (Markram et al., 1998). Complex time courses can result when Ca^{2+} binding to buffers and sequestration by pumps occur on the same time scale (Lee et al., 2000). Furthermore, saturation of Ca^{2+}-binding proteins can lead to nonlinear summation of Ca^{2+} signals and to changes in the range of action of Ca^{2+} signals (Allbritton et al., 1992; Maeda et al., 1999). For all these reasons accurate numbers are required for simulating and interpreting the observed properties of the Ca^{2+} signal. Given the fact that any cell may contain several Ca^{2+} buffer species (including the indicator dyes) and multiple extrusion mechanisms, the number of model parameters in a simulation is large. Therefore, being able to fix any one of them by an independent measurement is a blessing.

Naegerl et al. work with droplets of "flashing solution," which is a mixture of salts, a caged Ca^{2+} compound (DM-Nitrophen), and a low-affinity Ca^{2+} indicator dye. They measure free Ca^{2+} in the focal spot of a confocal microscope (with the resting beam) and elicit transients in free Ca^{2+} by photolysing DM-Nitrophen with a flash of UV light. They analyze the decay of $[Ca^{2+}]$ in the presence of Ca^{2+} buffers (first EGTA to validate the method, then calbindin-D28k) to determine kinetic rates of Ca^{2+} binding. The method should be applicable to intracellular measurements in any cell type that can be loaded with Ca^{2+} indicator dyes and caged Ca^{2+}. This should complement other methods in which intracellular buffers are titrated by continuous release of Ca^{2+} from caged Ca^{2+} or by multiple flashes (Xu et al., 1997; Maeda et al., 1999).

Naegerl et al. find that calbindin-D28k Ca^{2+}-binding kinetics are best described by assuming two types of Ca^{2+}-binding sites that differ in affinity and rate constants by factors of 2 to 6. The binding rate constants ($\sim$1 and $8 \cdot 10^7\ M^{-1}\ s^{-1}$ for the two sites, respectively) are surprisingly slow, the first one being comparable to that of EGTA (a typical "slow" buffer) and the second one being only 6–8 times

Received for publication 13 October 2000 and in final form 13 October 2000.

0006-3495/00/12/2783/02 $2.00

faster. However, the value for EGTA obtained with this method ($1.05 \cdot 10^7$ M^{-1} s^{-1}) is somewhat fast compared to recent T-jump experiments under very similar conditions (Naraghi, 1997). Based on the comparison with EGTA, calbindin-D28k might be considered to be situated between fast and slow Ca^{2+} buffers. Its exact role in shaping the Ca^{2+} signal within a living cell will depend on its concentration and on influences exerted by other components of the intracellular milieu.

In cerebellar Purkinje cells, known to contain large amounts of calbindin-D28k, dendritic Ca^{2+} transients evoked by subthreshold parallel fiber stimulation were significantly affected when experiments were performed on calbindin-D28k-deficient (CB $-/-$) mice (Airaksinen et al., 1997). Peak amplitudes were clearly enhanced, demonstrating that binding of Ca^{2+} to calbindin-D28k was sufficiently fast to affect $[Ca^{2+}]_i$ within the first 100 ms after the influx of Ca^{2+}. Caged Ca^{2+} experiments on Purkinje cells have revealed a high concentration of a high-affinity Ca^{2+}-binding protein with an affinity similar to the numbers reported here (Maeda et al., 1999). It was tentatively assigned to calbindin-D28k, but was reported to have cooperative Ca^{2+} binding, with an estimated Hill coefficient between 2 and 4. A complication in the analysis of these results arises from the fact that Purkinje cells not only express high levels of calbindin-D28k, but also equally high concentrations of the "slow" mobile buffer parvalbumin. It will be interesting to apply the method of Naegerl et al. to such cells and to see how calbindin-D28k injected into different types of neurones of CB$-/-$mice will affect Ca^{2+} transients.

I thank B. Schwaller, Fribourg, for helpful suggestions regarding the manuscript.

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