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Review

Measuring the kinetics of calcium binding proteins with flash photolysis ☆

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ABSTRACT

Background: Calcium-binding proteins (CBPs) are instrumental in the control of Ca^{2+} signaling. They are the fastest players within the Ca^{2+} toolkit responding within microseconds to $[Ca^{2+}]$ changes. The CBPs compete for Ca^{2+} which plays a direct role in modulating Ca^{2+} transients and the resulting biochemical message. The kinetic properties of the CBPs have to be known to have a good understanding of Ca^{2+} signaling.
Scope of review: Most techniques used to measure binding kinetics are too slow to accurately determine the fast kinetics of most CBP. Furthermore, many CBPs bind Ca^{2+} in a cooperative way, which should be incorporated in the kinetic modeling. Here we will review a new ultra-fast *in vitro* technique for measuring Ca^{2+} binding properties of CBPs following flash photolysis of caged Ca^{2+}. Compartmental modeling is used to resolve the kinetics of fast cooperative Ca^{2+} binding to CBPs.
Major conclusions: Currently this technique has only been used to quantify the kinetics of three CBPs (calbindin, calretinin and calmodulin), but has already provided remarkable insights into the specific role that these kinetics in Ca^{2+} signaling.
General significance: The potential to gain novel insights into Ca^{2+} signaling by quantifying kinetics of other CBPs using this technique is very promising. This article is part of a Special Issue entitled Biochemical, biophysical and genetic approaches to intracellular calcium signaling.

1. Introduction

1.1. The role of calcium binding proteins in Ca^{2+} signaling

Calcium ions (Ca^{2+}) are the signaling particles that function in the largest variety of biological signaling pathways. In all eukaryotic cells, Ca^{2+} signals play a crucial messenger role in the regulation of many processes including neurotransmission, muscle contraction, metabolism, cytoskeleton dynamics, gene transcription, cell cycle and cell death. Some of these Ca^{2+} signals are highly localized within a cell, while others are more global. Moreover, the regulation of Ca^{2+} practically covers the whole temporal spectrum over which biological processes are modulated, from (sub) milliseconds to years [1,2]. Notably, some of the processes triggered by an increase in intracellular $[Ca^{2+}]$ in a given cell oppose one another. For example, neuronal growth cone outgrowth/exploration vs. growth cone retraction [3] and long-term potentiation (LTP) vs. long-term depression (LTD) [4,5] are diametrically opposed processes. It is remarkable how a simple ion can regulate cellular functions in such a multitude of ways. How can changes in intracellular $[Ca^{2+}]$ modify cellular signaling over such a broad spectrum of processes with distinct temporal and spatial outcomes [6]? Since the serendipitous discovery of Ca^{2+} as an essential signaling ion in 1883 by Ringer [7] many strides have been made towards answering this question.

The structure of a Ca^{2+} signal can generally be described as follows: at resting conditions the intracellular $[Ca^{2+}]$ is kept low, around 100 nM. Upon an appropriate Ca^{2+} stimulus, so-called ON-mechanisms are activated [1,2]. These ON-mechanisms (e.g., voltage- or ligand-gated ion channels in the plasma membrane or IP3 activated channels) let Ca^{2+} into the cytoplasm from the extracellular space or intracellular organelles (Ca^{2+} stores, i.e. sarco-endoplasmatic reticulum or mitochondria) causing a rapid increase in cytosolic $[Ca^{2+}]$. If this 'signal' is sufficiently large it will be 'translated' into a biochemical message. When the $[Ca^{2+}]$ increases sufficiently, Ca^{2+} will bind to sensor Ca^{2+} binding proteins (CBPs), changing the physiological properties of these proteins. A hallmark for these sensor CBPs is a relatively large conformational change upon Ca^{2+} binding that is often accompanied by exposure of hydrophobic surfaces. Consequently, this allows interactions with specific ligands linked to subsequent biochemical regulation of downstream effectors [8]. Meanwhile, OFF-mechanisms work to lower the $[Ca^{2+}]$ in the cytoplasm to the resting concentrations [1,2]. These OFF-mechanisms are the pumps and exchangers that transport the Ca^{2+} either back into the Ca^{2+} stores or to the extracellular space. Furthermore, there are buffering CBPs that rapidly bind free Ca^{2+}, causing a seemingly immediate decrease in $[Ca^{2+}]$. However, in later phases of the OFF-period, as the $[Ca^{2+}]$ decreases, these CBPs will release the bound Ca^{2+}, causing a prolonged Ca^{2+} signal. Therefore, CBPs are not

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strictly OFF-mechanisms, but they are involved in shaping the amplitude and duration of the Ca^{2+} signal [1,2,9,10]. According to its specific function, each cell expresses a unique and specific set of Ca^{2+} signaling tools, i.e., ON and OFF components, to create the distinctive spatial and temporal Ca^{2+} signaling properties needed for the cell's function [11]. Depending on their exact composition of their Ca^{2+} signaling toolkit, each Ca^{2+} signal system will produce Ca^{2+} transients varying from milliseconds to several hundreds of milliseconds. Various names are given to the Ca^{2+} transients, such as sparks, embers, quarks, puffs, blips or waves, depending on their exact spatial and temporal properties and the cell type in which they occur [2,12]. Furthermore, the Ca^{2+} signals can be highly repetitive, forming Ca^{2+} oscillations [13]. In short, the spatiotemporal characteristics of short-lived and often highly localized changes in intracellular $[Ca^{2+}]$ result from a complex interplay between Ca^{2+} influx/extrusion systems, mobile/stationary CBPs, and intracellular sequestering mechanisms.

To understand the kinetics of cellular Ca^{2+} transients and their influence on the processes they regulate requires an in-depth knowledge of the Ca^{2+} sensitivities and binding properties of all the components involved. Upon an increase in $[Ca^{2+}]$, the CBPs are the first to respond as they immediately start binding Ca^{2+}. Within each system, a Ca^{2+} signal will be interpreted and translated depending on the amplitude and temporal pattern of Ca^{2+} binding to the sensor CBPs. Both buffering and sensing CBPs are the fastest players within the Ca^{2+} toolkit and respond directly, working on a timescale of tens of microseconds to tens of milliseconds. The various CBPs are in an immediate competition to bind the freshly available Ca^{2+}. On the other hand, the OFF components work on a somewhat slower timescale of tens of milliseconds to seconds and will not immediately reduce the $[Ca^{2+}]$ back to normal [14]. Hence, the competition for Ca^{2+} between the various CBPs within a system plays an essential and direct role in modulating the shape of Ca^{2+} transients and the outcome of the conveyed biochemical message [10]. Evidently, to have a good understanding of Ca^{2+} signaling, it is essential to know the properties of the CBPs that are involved in the studied process. A few key features of CBPs determine the spatiotemporal characteristics of Ca^{2+} signals and their transduction: the overall Ca^{2+} affinity of CBPs, their localization and concentration, their mobility inside cells, and their binding kinetics [15]. The latter of which are conceivably the most critical determinant of cellular Ca^{2+} signaling [16]. The lack of accurate data on the kinetic properties of CBPs gives rise to uncertainties in models studying intracellular Ca^{2+} signaling [10]. Two major obstacles make it challenging to accurately determine the kinetic properties of CBPs. First, the Ca^{2+} binding kinetics are very fast and, for accurate quantification, require the ability to measure changes in $[Ca^{2+}]$ (or any other parameter related to Ca^{2+} binding) with an accuracy of 10–100 μs. Conventional techniques used to measure binding kinetics to macromolecules, like stopped flow fluorimetry, have dead times>1 ms [17], precluding accurate determination of the faster Ca^{2+} binding kinetics of CBPs. Secondly, many of the CBPs bind Ca^{2+} in a cooperative way, which is the ability to influence ligand binding at a site of a macromolecule by previous ligand binding to another site of the same macromolecule. There are four commonly used descriptions for cooperativity (for review see [18]): the Hill [19], the Adair-Klotz [20,21], the Monod-Wyman-Changeux (MWC) [22], and the Koshland–Némethy–Filmer (KNF) [23] models. Yet all these models describe cooperativity only when the binding reactions are at equilibrium. Only under specific conditions [24–26] the MWC model was used to describe kinetics of cooperative binding. Such conditions do not hold for Ca^{2+} binding to CBPs. Furthermore, when using the MCW model with most CBPs the mathematical description becomes too complex for simple/practical interpretations [18,27]. Over the last few years we have been working on overcoming these obstacles. We have developed an *in vitro* technique to measure the fast Ca^{2+}-binding kinetics CBPs following flash photolysis of caged Ca^{2+} [28,29]. In combination with compartmental kinetic modeling and a simple kinetic model for cooperative binding, we have begun to resolve the Ca^{2+} binding kinetics of some CBPs [28,30,31]. In this paper we will give a short overview some of the commonly used techniques that give insight into the Ca^{2+} binding kinetics of CBPs. We will then discuss our technique and describe various findings we have discovered while developing our technique that may be relevant to others using similar methods, such as measuring $[Ca^{2+}]$ with fluorescent dyes.

1.2. Ca^{2+} buffering capacity (κ) is a description of Ca^{2+} binding kinetics

One of the practical ways to quantify Ca^{2+} buffering in a cell is the buffering capacity (κ), which is the ratio of buffer-bound Ca^{2+} to free Ca^{2+} upon a change in total Ca^{2+} [32,33]:

$$\kappa_S = \frac{d[CaS]}{d[Ca^{2+}]}$$

where S is the endogenous buffer. For example, if $\kappa_s = 23$, then out of every 24 ions entering a compartment, 23 will be bound by S (i.e., ~4% of Ca^{2+} entering remains unbound). This number gives important insights into several aspects of Ca^{2+} signaling. For instance, the size of the Ca^{2+} influx required to reach a certain free $[Ca^{2+}]$ can be determined using κ. As it is defined in the equation above κ does not reveal anything about the dynamics of Ca^{2+} buffering. However, the κ that is generally used in literature does. It is not trivial to determine the *theoretical* κ in a (sub)cellular compartment because it requires a small *known* change in $[Ca^{2+}]_{total}$ and a measurement of the resulting Ca^{2+} signal. To evoke a change in intracellular $[Ca^{2+}]$, one can stimulate the cell to open Ca^{2+} permeable channels. In many structures, such as dendritic spines, it is impossible to use a technique (e.g., voltage clamping) to precisely determine the evoked Ca^{2+} influx (e.g., by measuring the Ca^{2+} current). Hence, it is impossible to exactly determine the amount of Ca^{2+} entering the structure. Estimates may be made based on the number of expected open channels and the driving force for Ca^{2+} over the whole time course of the Ca^{2+} influx. But unfortunately, often there are no exact data on the number of Ca^{2+} permeable channels open following stimulation, or the exact time-course of the membrane potential, hence the driving force for Ca^{2+}. Another approach is to measure the Ca^{2+} signal following the Ca^{2+} influx by using Ca^{2+}-indicators such as fura-2 or Oregon Green BAPTA (OGB). However, these dyes act as exogenous Ca^{2+} buffers, which will have a significant impact on the Ca^{2+} signal itself. Therefore, an approach has been developed that gives a quantification of the buffer capacity of endogenous buffers by extrapolating a series of indicator concentrations to zero [32,34,35]. It can be derived that changes in $[Ca^{2+}]$ at equilibrium:

$$\Delta\left[Ca^{2+}\right]_{(t=\infty)} = \frac{\Delta\left[Ca^{2+}\right]_{total(t=\infty)}}{1+\kappa_B+\kappa_S}.$$

If Ca^{2+} binding to all the buffers (endogenous S, and exogenous B) is fast enough so that the binding reactions are always close to equilibrium, then by approximation:

$$\Delta\left[Ca^{2+}\right]_{(t)} = \frac{\Delta\left[Ca^{2+}\right]_{total(t)}}{1+\kappa_B+\kappa_S}.$$

The assumption that the buffers are fast enough to always be in equilibrium (i.e., easily follow the $[Ca^{2+}]$ increase induced by the ON-systems) automatically implies that

$$\Delta\left[Ca^{2+}\right]_{peak} = \frac{\Delta\left[Ca^{2+}\right]_{total}}{1+\kappa_B+\kappa_S}.$$