Topic Introduction

Measuring the Ca^{2+} -Binding Kinetics of Proteins

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Ca^{2+} -binding proteins (CBPs) are instrumental in the control of Ca^{2+} signaling. For the transduction of a change in intracellular Ca^{2+} concentration into a cellular biochemical or biophysical action, it is necessary for Ca^{2+} to bind specific Ca^{2+} -binding proteins (CBPs) that relay the Ca^{2+} signal. The competition for Ca^{2+} between the various CBPs plays an essential and direct role in this transduction and in the resulting biochemical message. Therefore, a thorough understanding of Ca^{2+} signaling necessitates appreciating the kinetic properties of all the relevant CBPs. Unfortunately, most conventional techniques used to measure Ca^{2+} -binding kinetics are too slow to determine accurately the fast binding kinetics of most CBPs. To address this problem, we have developed an ultrafast in vitro technique for measuring the Ca^{2+} -binding properties of CBPs following flash photolysis of caged Ca^{2+} . We introduce here the protocols that are necessary for the data collection associated with this technique.

INTRODUCTION

The entry of Ca^{2+} ions through the plasma membrane controls intercellular communication in virtually all living species. The signal is encoded by a change in intracellular calcium concentration [Ca^{2+}]. Transduction of this signal consists of Ca^{2+} binding to signaling Ca^{2+} -binding proteins (sCBPs). This Ca^{2+} binding causes a biophysical change in the sCBPs (most often a conformational change) that translates into downstream biochemical or biophysical actions (Ikura 1996). Next to these sCBPs, many cell types also contain buffering CBPs (bCBPs). These bCBPs bind Ca^{2+} without directly triggering a biochemical signal—hence, they just serve to buffer the [Ca^{2+}].

All CBPs present in a cell will compete for the Ca^{2+} , and this competition determines the downstream effect of a given Ca^{2+} signal. On a static change in [Ca^{2+}], a CBP with a high affinity for Ca^{2+} will eventually bind relatively more Ca^{2+} than one with a low affinity. However, if the low-affinity CBP has a much faster association rate for Ca^{2+} , it might temporarily outcompete the high-affinity CBP and postpone the binding to the high-affinity CBP (Faas et al. 2011). Ca^{2+} signals are highly dynamic—that is, the Ca^{2+} signal is not a step in the [Ca^{2+}] from one value to another but, rather, an increase in [Ca^{2+}] that is highly temporally and spatially modulated, never reaching a steady state.

CHALLENGES IN STUDYING Ca^{2+} -BINDING KINETICS

To understand how and why Ca^{2+} signals are translated into a certain biochemical or biophysical response, it is essential to know the specific Ca^{2+} -binding properties of all the CBPs in a signal

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transduction pathway. The dynamic properties of the CBPs—that is, the Ca^{2+} binding rate constants (k_{on} and k_{off} values), and not their steady-state affinities—are some of the most crucial properties determining the downstream effect of a Ca^{2+} signal (Williams 1994; Neher 2000, 2008; Faas et al. 2007, 2011; Faas and Mody 2012).

Measuring the kinetics of CBPs is quite challenging for two reasons. First, the process of Ca^{2+} binding to CBPs is very fast—for example, the k_{on} values for Ca^{2+} binding to calmodulin are in the order of 10^7 – $10^{10} \text{ M}^{-1}\text{sec}^{-1}$ (Faas et al. 2011). The precise measurement of these properties requires the ability to measure changes in $[\text{Ca}^{2+}]$ (or any other parameter related to Ca^{2+} binding) with an accuracy of 10–100 μsec . Conventional techniques used to measure binding kinetics to macromolecules, such as stopped-flow fluorimetry, have dead times $>1 \text{ msec}$ (Bisswanger 2002), precluding accurate determination of the fast (submillisecond) Ca^{2+} -binding kinetics of CBPs. Second, many of the CBPs bind Ca^{2+} in a cooperative manner that is mathematically challenging to include in the kinetic modeling.

A METHOD FOR MEASURING Ca^{2+} -BINDING KINETICS

With the above limitations in mind, we have developed an in vitro technique to measure the fast Ca^{2+} -binding kinetics of CBPs following flash photolysis of caged Ca^{2+} . 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. For example, with this technique, we were able to quantify the kinetics of calmodulin, which can bind Ca^{2+} with a speed that is diffusion limited ($\sim 10^{10} \text{ M}^{-1}\text{sec}^{-1}$), and incorporated its cooperative nature of Ca^{2+} binding ($n_{\text{H}} \sim 1.9$ for two pairs of binding sites) into a kinetic model (Faas et al. 2011).

FIGURE 1. Scheme of all equilibrium reactions occurring in the measurement chamber after photolysis of the caged- Ca^{2+} compound DM-nitrophen (DMn). The rate constants for DMn, its photoproducts (PP) and the uncaging time constants (τ_{f} and τ_{s}) of DMn are independently determined for each experiment (also see **Collecting Data to Determine the Ca^{2+} Binding Properties of DM-Nitrophen and Proteins** [Faas and Mody 2014c]). The rate constants and the F_{ratio} for each batch of OGB-5N are also measured in an independent experiment (see **Measuring the Steady-State Properties of Ca^{2+} Indicators with a Set of Calibrated $[\text{Ca}^{2+}]$ Solutions** [Faas and Mody 2014a] and **Measuring the Rate Constants of Ca^{2+} Indicators** [Faas and Mody 2014b]). The reaction parameters to be determined for describing the Ca^{2+} binding to a CBP are indicated by question marks. The model/scheme for the buffering reaction is dependent on the CBP used and is represented here by the simple equilibrium between $[\text{CBP}]$ and $[\text{Ca}_n\text{CBP}]$ —but this can become quite complex (Faas et al. 2007, 2011). Figure adapted from Figure 1 in an article published previously (Faas et al. 2007).

A simplified description of this technique is as follows: In a reaction chamber (cuvette), there is a mixture of the CBP to study, a caged- Ca^{2+} compound such as DM-nitrophen (DMn), and a fluorescent Ca^{2+} indicator (or dye, D) such as Oregon Green BAPTA 5N (OGB-5N). At the start of the experiment, the $[\text{Ca}^{2+}]_{\text{free}}$ is low, but most of the caged- Ca^{2+} compound has Ca^{2+} bound. Then, with a short flash of UV light, a part of the caged- Ca^{2+} compound is photolyzed and breaks into photoproducts. These photoproducts have a much lower affinity for Ca^{2+} than the original caged- Ca^{2+} compound and will release most of the Ca^{2+} bound, resulting in a rapid increase of the $[\text{Ca}^{2+}]_{\text{free}}$. This Ca^{2+} will then bind to the CBP and the Ca^{2+} indicator, lowering the $[\text{Ca}^{2+}]_{\text{free}}$. The changes in $[\text{Ca}^{2+}]_{\text{free}}$ are measured by recording the fluorescence of the Ca^{2+} indicator. The fluorescence data are then fitted with a mathematical model that describes all the reactions in the reaction chamber, as depicted in Figure 1.

Details of the setup, the mathematics, and the analysis involved in this technique have been described elsewhere (Nagerl et al. 2000; Faas et al. 2005, 2007, 2011; Faas and Mody 2012). However, many practical and technical details that involve the actual measurements are described only in minimally necessary detail. We provide two protocols for accurately quantifying the properties of a Ca^{2+} indicator (see **Measuring the Steady-State Properties of Ca^{2+} Indicators with a Set of Calibrated $[\text{Ca}^{2+}]$ Solutions** [Faas and Mody 2014a] and **Measuring the Rate Constants of Ca^{2+} Indicators** [Faas and Mody 2014b]). Furthermore, we provide a detailed protocol explaining how to prepare solutions that contain a caged- Ca^{2+} compound and a specific starting $[\text{Ca}^{2+}]_{\text{free}}$. In this latter protocol (**Collecting Data to Determine the Ca^{2+} Binding Properties of DM-Nitrophen and Proteins** [Faas and Mody 2014c]), we also describe the procedure for data collection. We hope that adhering to these procedures will eliminate most of the practical hurdles involved in collecting experimental data for those who want to use our technique or one similar to it.

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