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Review

Dynamic visualization of calcium-dependent signaling in cellular microdomains

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ABSTRACT

Cells rely on the coordinated action of diverse signaling molecules to sense, interpret, and respond to their highly dynamic external environment. To ensure the specific and robust flow of information, signaling molecules are often spatially organized to form distinct signaling compartments, and our understanding of the molecular mechanisms that guide intracellular signaling hinges on the ability to directly probe signaling events within these cellular microdomains. Ca^{2+} signaling in particular owes much of its functional versatility to this type of exquisite spatial regulation. As discussed below, a number of methods have been developed to investigate the mechanistic and functional implications of microdomains of Ca^{2+} signaling, ranging from the application of Ca^{2+} buffers to the direct and targeted visualization of Ca^{2+} signaling microdomains using genetically encoded fluorescent reporters.

1. Introduction

All living cells must continually sense and respond to changes in their external chemical environment. During intracellular signaling, information regarding the conditions outside the cell is passed along from the cell surface to the appropriate response machinery inside the cell. Yet although cells contain diverse signaling pathways that specifically control the myriad biological processes that are essential to life, the pool of signaling molecules that comprise these pathways is limited, and specificity cannot be intrinsically encoded into individual pathways. Rather, in order to both ensure the specificity and promote the diversity of signaling outcomes, cells must carefully coordinate the actions of signaling molecules as they participate in a dynamic network of highly integrated signaling pathways. One way for cells to achieve this level of coordination is through the spatial compartmentalization of the cell interior into local signaling domains of various sizes, and intracellular Ca^{2+} signaling offers a striking example of this process.

Ca^{2+} signaling regulates many fundamental biological processes, including neurotransmission, muscle contraction, gene expression, cell proliferation, and cell death [1], often regulating multiple cellular processes in parallel. The remarkable versatility of Ca^{2+} as an intracellular messenger stems from the exquisite spatial and temporal regulation of elevations in intracellular Ca^{2+} concentrations, especially through the formation of discrete microdomains of Ca^{2+} signaling. Often, these microdomains involve so-called "elementary" Ca^{2+}-release events that result from the opening of individual Ca^{2+} channels [2]. Broadly speaking, however, the term Ca^{2+} microdomain can apply not only to zones of high Ca^{2+} concentration that occur near the mouths of Ca^{2+} channels but to any Ca^{2+} signaling event that is confined to a particular region of the cell – be it the plasma membrane, a specific part of the cytosol, or Ca^{2+}-storing organelles – as opposed to the cytoplasm as a whole [3,4].

As with the compartmentalization of other signaling molecules, such as cAMP (see [5]), the existence of Ca^{2+} microdomains was originally proposed to account for experimental observations that lacked a clear mechanistic foundation. Various theoretical studies (e.g., [6,7]) and indirect observations [8] bolstered this idea, but it was not until the development of optical (e.g., fluorescent) techniques to visualize Ca^{2+} dynamics in intact cells that direct experimental evidence of Ca^{2+} microdomains was obtained. Following the adoption of fluorescent probes to study local Ca^{2+} dynamics, the more recent development of genetically encoded fluorescent reporters based on green fluorescent protein (GFP) and related fluorescent proteins has completely revolutionized the

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study of spatially confined signaling events. In this review, we provide a brief primer on the design and development of genetically encoded fluorescent reporters and discuss the application of these biosensors to the study of spatially compartmentalized signaling events in living cells, using Ca^{2+} signaling as an example.

2. Fluorescent biosensors

Understanding the functional and mechanistic properties of signaling microdomains depends on our ability to assay molecular events specifically within these confined cellular regions. In other words, the more selectively we can target these microdomains, the more precisely we can study local signaling. These efforts have been greatly facilitated by advances in live-cell fluorescence imaging approaches, which have deep ties to the study of Ca^{2+} signaling. For example, the use of fluorescent indicator dyes for live-cell imaging was popularized by the success of Ca^{2+} indicators such as quin-2, fluo-3, and fura-2 (Fig. 1A) [9], and it was the isolation of the Ca^{2+}-dependent photoprotein aequorin – itself a useful tool for monitoring Ca^{2+} inside cells (Fig. 1B) [10,11] – that ultimately led to the discovery of *Aequoria victoria* GFP (reviewed in [12,13]) and the development of genetically encoded fluorescent reporters. These genetically encoded molecular tools can easily be expressed in cells using recombinant DNA technology and can further be targeted throughout the cell via the incorporation of endogenous subcellular targeting motifs or via fusion to endogenous proteins that natively localize to specific regions of the cell. This relatively straightforward targetability enables the specific and detailed investigation of signaling processes occurring within all manner of cellular microdomains and compartments [14,15].

Genetically encoded fluorescent reporters come in a variety of forms (reviewed extensively in [16]) that all share a highly generic, modular design in which a sensing unit capable of detecting a specific biochemical signal is coupled to a reporting unit composed of one or more fluorescent proteins. In the most versatile class of biosensors developed thus far, the sensing unit encompasses a protein or protein fragments in the form of a molecular switch that undergoes a conformational change in response to a particular input signal. The conformational change in the molecular switch then alters the distance and/or orientation of a pair of fluorescent proteins capable of undergoing Förster resonance energy transfer (FRET) [17]. The first such FRET-based biosensor, cameleon, was generated by sandwiching the Ca^{2+} sensor calmodulin (CaM) and a CaM-binding peptide from myosin light-chain kinase (M13) between a FRET pair [18,19], again highlighting the close ties between Ca^{2+} signaling and live-cell fluorescence imaging. In the presence of Ca^{2+}, CaM binds the M13 peptide and induces a conformational change that alters the FRET signal from cameleon (Fig. 1C). In an alternative design that eschews FRET, CaM and M13 are instead inserted within a single fluorescent protein and used to directly control the fluorescence intensity, as in the popular GCaMP series of Ca^{2+} sensors (Fig. 1D) [20,21].

Molecular switches have proven to be an extremely versatile solution to the problem of monitoring signaling in living cells, as they are readily adaptable to detecting a variety of biochemical events associated with signaling. As demonstrated with cameleon, molecular switches can often be engineered from protein components that will interact and induce a conformational change in response to a given signal. These bipartite switches comprise a receiving domain that is modified by the input signal and a switching domain that interacts with the receiver to drive the conformational change (Fig. 1E). For example, numerous kinase activity reporters have been generated by fusing a consensus substrate sequence and a phosphoamino acid-binding domain (PAABD), which are then sandwiched between a FRET pair [22]. Binding of the phosphorylated substrate by the PAABD leads to a conformational change that produces a FRET change. Similarly, fusing a small GTPase to a binding partner that only interacts with the active, GTP-bound form of the enzyme results in a molecular switch that underlies many GTPase activation sensors [23]. Molecular switches can also be derived from the intrinsic conformational dynamics of native proteins (Fig. 1E). The most commonly used cAMP biosensors, for instance, use the intrinsic conformational change that occurs when cAMP binds exchange protein activated by cAMP (Epac) to drive a FRET change [24,25]. In theory, any protein whose conformation is directly modulated by an upstream signal can be inserted between a FRET pair to construct a biosensor, though this frequently involves trial and error. This generalizable design scheme has inspired the development of a multitude of biosensors capable of detecting a broad spectrum of biochemical processes (e.g., second messenger production, ion and metabolite concentrations, enzyme activity, and enzyme activation), making genetically encoded fluorescent reporters an invaluable asset in the study of intracellular signaling [15,16].

3. Investigating microdomains of Ca^{2+} signaling

Many approaches have been employed over the years to investigate local Ca^{2+} signaling processes within cells. These range from indirect measurements based on Ca^{2+} buffering to highly selective, direct visualization of Ca^{2+} signals using genetically encoded biosensors. As more targeted and versatile methods have become available, the ability to probe Ca^{2+} signaling has also expanded to include not only Ca^{2+} itself but also a number of Ca^{2+}-dependent signaling proteins, thus fueling the development of a much more sophisticated understanding of Ca^{2+} signaling microdomains.

3.1. Ca^{2+} microdomains at the plasma membrane

Ca^{2+} influx across the plasma membrane is an essential signaling mechanism in all cell types, especially in electrically excitable cells such as neurons and muscle cells. Hence, early studies of local Ca^{2+} signaling often focused on Ca^{2+} microdomains near the plasma membrane. Plasma membrane Ca^{2+} microdomains were initially proposed to help explain the very rapid release of neurotransmitter in response to Ca^{2+} influx, under the assumption that the exocytotic machinery must be closely coupled to Ca^{2+} channels [26]. The existence of these microdomains could be observed indirectly by loading cells with different chelators to buffer global Ca^{2+} increases [8]. Typically, cells are loaded with either the "slow" chelator EGTA or the "fast" chelator BAPTA, the idea being that given a very short (<100 nm) distance between the Ca^{2+} channel and the target, only BAPTA will be able to capture Ca^{2+} quickly enough to disrupt the microdomain (Fig. 2A) [8,27]. This is a powerful technique that is still used to study Ca^{2+} microdomains in the vicinity of plasma membrane Ca^{2+} channels to this day. For example, both BAPTA and EGTA were recently shown to disrupt transmission in mature hippocampal mossy fiber synapses, suggesting that synaptic vesicles are only loosely coupled to Ca^{2+} channels in these plastic synapses, in contrast to the tight coupling that is often observed in the mature nervous system [28]. In addition, Selway et al. recently used cell-permeable versions of these chelators to demonstrate that Ca^{2+} microdomains formed by L-type voltage-gated Ca^{2+} channels (VGCCs) were sufficient to activate ERK signaling in response to GLP-1 stimulation in MIN6 pancreatic β-cells [29].

Detailed studies of Ca^{2+} microdomains, however, require more direct methods for monitoring local Ca^{2+} signaling events in cells. A number of optical detection methods have been developed to permit the direct visualization of intracellular Ca^{2+} dynamics in living cells, and many of these had already begun to see widespread adoption by the late 1980s [30]. In many cases, these diffusible probes

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