This Review is part of a thematic series on Imaging of Cardiovascular Cells and Tissues, which includes the following articles:

- Use of Chimeric Fluorescent Proteins and Fluorescence Resonance Energy Transfer to Monitor Cellular Responses
- Optical Imaging of the Heart
- Examining Intracellular Organelle Function Using Fluorescent Probes
- Two-Photon Microscopy of Cells and Tissues

Brian O’Rourke, Guest Editor

Imaging Microdomain Ca\(^{2+}\) in Muscle Cells

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Abstract—Ca\(^{2+}\) ions passing through a single or a cluster of Ca\(^{2+}\)-permeable channels create microscopic, short-lived Ca\(^{2+}\) gradients that constitute the building blocks of cellular Ca\(^{2+}\) signaling. Over the last decade, imaging microdomain Ca\(^{2+}\) in muscle cells has unveiled the exquisite spatial and temporal architecture of intracellular Ca\(^{2+}\) dynamics and has reshaped our understanding of Ca\(^{2+}\) signaling mechanisms. Major advances include the visualization of “Ca\(^{2+}\) sparks” as the elementary events of Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR), “Ca\(^{2+}\) sparklets” produced by openings of single Ca\(^{2+}\)-permeable channels, miniature Ca\(^{2+}\) transients in single mitochondria (“marks”), and SR luminal Ca\(^{2+}\) depletion transients (“scraps”). As a model system, a cardiac myocyte contains a 3-dimensional grid of 10\(^4\) spark ignition sites, stochastic activation of which summates into global Ca\(^{2+}\) transients. Tracking intermolecular coupling between single L-type Ca\(^{2+}\) channels and Ca\(^{2+}\) sparks has provided direct evidence validating the local control theory of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in the heart. In vascular smooth muscle myocytes, Ca\(^{2+}\) can paradoxically signal both vessel constriction (by global Ca\(^{2+}\) transients) and relaxation (by subsurface Ca\(^{2+}\) sparks). These findings shed new light on the origin of Ca\(^{2+}\) signaling efficiency, specificity, and versatility. In addition, microdomain Ca\(^{2+}\) imaging offers a novel modality that complements electrophysiological approaches in characterizing Ca\(^{2+}\) channels in intact cells. (Circ Res. 2004;94:1011-1022.)

Key Words: Ca\(^{2+}\) signaling • Ca\(^{2+}\) sparks • Ca\(^{2+}\) channels • excitation-contraction coupling • sarcoplasmic reticulum

As a ubiquitous intracellular messenger, Ca\(^{2+}\) plays critical roles in a myriad of physiological processes, including muscle contraction, synaptic transmission, hormone secretion, gene transcription, cell survival, and cell death.\(^1\)\(^-\)\(^5\) Ca\(^{2+}\) as an inorganic divalent cation exists in only one chemically and biologically relevant form and is diffusible in the cytosolic milieu. A fundamental issue has arisen as to how the same ion can fulfill different and even opposing physiological functions in a given cell. Over the last decade, it has been increasingly appreciated that spatial and temporal patterning endows Ca\(^{2+}\) signaling with efficiency, specificity, and unparalleled versatility. Optical visualization of microdomain (0.1 to 10 \(\mu\)m) Ca\(^{2+}\) thus holds the promise to dissect out the space-time architecture of Ca\(^{2+}\) dynamics and, thereby, to unravel Ca\(^{2+}\) signaling mechanisms in different physiological contexts.

Since the first recording of Ca\(^{2+}\) sparks\(^6\) in cardiac myocytes in 1993, imaging microdomain Ca\(^{2+}\) in muscles has
Select Terminology of Ca\(^{2+}\) Signaling Events

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<th>Term</th>
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<td>CRU</td>
<td>A Ca(^{2+}) release unit (CRU) refers to a group of Ca(^{2+}) release channels (RyRs or IP3Rs or both) clustered in the ER/SR membrane(^{62})</td>
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<td>Ca(^{2+}) sparks</td>
<td>Event or the optical image of Ca(^{2+}) release from a single CRU(^{6})</td>
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<td>Ca(^{2+}) spiklet</td>
<td>Event or the optical image of Ca(^{2+}) fluxing through a single Ca(^{2+})-permeable channel(^{75})</td>
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<td>Compound Ca(^{2+}) sparks</td>
<td>Event or the optical image of near-synchronous activation of multiple adjacent CRUs(^{43})</td>
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<td>Ca(^{2+}) puff</td>
<td>Synonym to Ca(^{2+}) spark or compound Ca(^{2+}) spark when a single or multiple CRUs of IP(_3)R are involved(^{46})</td>
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<tr>
<td>Ca(^{2+}) quark</td>
<td>Synonym to RyR Ca(^{2+}) spiklet(^{63})</td>
</tr>
<tr>
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<td>Ca(^{2+}) spike</td>
<td>(1) Spatially averaged Ca(^{2+}) transient measured in the presence of excessive Ca(^{2+}) buffers. In cardiac myocytes, it mainly reflects SR Ca(^{2+}) release function(^{41})</td>
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<td>(2) Local Ca(^{2+}) transient measured in the presence of excessive Ca(^{2+}) buffers. In cardiac myocytes, it mainly reflects Ca(^{2+}) release function underlying a single or compound Ca(^{2+}) spark at a Z-line/T-tubule site(^{61})</td>
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offered unique insights into the molecular mechanisms of excitation-contraction (EC) coupling and revolutionized our understanding of Ca\(^{2+}\)-mediated signal transduction (see reviews\(^{2,5,7-19}\)). In this review, we intend to summarize advances in this rapidly evolving field, with an emphasis on cardiac microdomain Ca\(^{2+}\) imaging. In keeping with the aims of this review series, pertinent technical aspects will be discussed in an attempt to identify merits, potentials, and limitations of the various Ca\(^{2+}\) imaging techniques used. Selected terminologies used to describe cellular signaling Ca\(^{2+}\) events are listed in the Table.

\[\text{Ca}^{2+}\] Gradients in Living Cells: Theoretical Considerations

Sustained gradients of free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) can be found between membrane-delimited cellular and subcellular compartments. A 10\(^{5}\)-fold [Ca\(^{2+}\)] gradient exists across the plasma membrane as well as the membrane of the endoplasmic and sarcoplasmic reticulum (ER/SR). The cytosolic [Ca\(^{2+}\)] is actively maintained at a very low level around 100 nmol/L, by a system of Ca\(^{2+}\) homeostatic regulatory mechanisms, including the ER/SR Ca\(^{2+}\) ATPase and the sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchanger and Ca\(^{2+}\) ATPase. The signal Ca\(^{2+}\) can then be rapidly mobilized, often via Ca\(^{2+}\)-permeable channels, from the exterior or the intracellular Ca\(^{2+}\) reservoirs, resulting in [Ca\(^{2+}\)] transients in the cytosol.

At one time, the cytosolic Ca\(^{2+}\) was conceptualized as a “common pool” in which [Ca\(^{2+}\)] rises and falls uniformly. A corollary of this notion is that Ca\(^{2+}\) signaling in a given cell is fully determined by the magnitudes and the temporal dynamics of spatially averaged [Ca\(^{2+}\)]. At the molecular level, however, transmembrane Ca\(^{2+}\) translocation is a discrete process mediated by Ca\(^{2+}\)-permeable channels and Ca\(^{2+}\) transporters. When in action, these molecular entities function as either Ca\(^{2+}\) sources (admitting Ca\(^{2+}\) into a subcellular compartment) or Ca\(^{2+}\) sinks (removing Ca\(^{2+}\) from the compartment) and create dynamic [Ca\(^{2+}\)] gradients in their immediate vicinity. Theoretically,\(^{11-15}\) many cellular and molecular determinants shape the microscopic [Ca\(^{2+}\)] gradients. These include (1) unidirectional Ca\(^{2+}\) fluxes, (2) intracellular Ca\(^{2+}\) buffers, many of which are also Ca\(^{2+}\) effectors, and (3) Ca\(^{2+}\) diffusion in the cytosolic milieu. In addition, [Ca\(^{2+}\)] gradients will be accentuated and confined by spatial restriction, eg, disc-shaped dyadic clefts,\(^{16,17}\) tortuous ER/SR network, or membrane-bound subcellular organelles such as mitochondria. The physiological relevance of such Ca\(^{2+}\) microdomains had not been fully appreciated until about a decade ago, when they were first visualized and investigated experimentally.

Imaging the Elementary Intracellular Ca\(^{2+}\) Release Events

Ca\(^{2+}\) signals in living cells were first “seen” in the late 1960s as [Ca\(^{2+}\)] transients from single twitch barnacle muscle fibers\(^{18}\) and first imaged in the late 1970s as [Ca\(^{2+}\)] waves in fertilizing medaka fish eggs.\(^{19}\) In both cases, [Ca\(^{2+}\)] was measured by means of chemiluminescent aequorin. Shortly after, [Ca\(^{2+}\)] transients during cardiac EC coupling were seen as aequorin signals in canine Purkinje fibers.\(^{20}\) Measurement of [Ca\(^{2+}\)] transients began to seriously expand with metallochromic dyes, first arsenazo III, used by Brown et al in squid axon\(^{21}\) and then in muscle,\(^{22}\) and later antipyrylazo III used by Kovacs et al.\(^{23}\) In heart muscle cells, the use of fluorescent reporter, fura-2, was first introduced in conjunction with digital imaging to document spontaneous [Ca\(^{2+}\)] waves by Wier et al.\(^{24}\) With the advent of confocal microscopy and the new generation of fluorescent indicator, fluo-3, microdomain Ca\(^{2+}\) in muscles was first visualized as Ca\(^{2+}\) sparks in quiescent cardiac myocytes (Figure 1A) in 1993.\(^{5}\)

A Ca\(^{2+}\) spark appears abruptly amid a seemingly featureless background, reaches its peak within 10 ms, and dissipates in another 20 ms; it reflects the spontaneous activation of ryanodine receptors (RyRs)\(^{25,26}\) in a single Ca\(^{2+}\) release unit (CRU) in the ER/SR. In spite of the Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) mechanism\(^{27-29}\) operating in these cells, a Ca\(^{2+}\) spark normally remains solitary and is confined to an area of \(\approx 2.0 \mu m\) in diameter. Under Ca\(^{2+}\) overload conditions, however, a Ca\(^{2+}\) spark can sometimes activate release from neighboring CRUs to form a compound spark\(^{30-32}\); ordered spatiotemporal activation of sparks and compound sparks can then evolve into propagating [Ca\(^{2+}\)] waves.\(^{5,31}\)

Ca\(^{2+}\) sparks with identical properties can be evoked by Ca\(^{2+}\) influx through voltage-operated L-type Ca\(^{2+}\) channels (LCCs), known also as dihydropyridine receptors (DHPRs),
via the CICR mechanism (Figure 1B).33–37 To resolve individual events, Ca$^{2+}$ sparks should be activated at a low density, by near-threshold or very brief depolarization,35,37 with reduced extracellular [Ca$^{2+}$].38 or under conditions where LCC availability is reduced pharmacologically.35–40 Both the trigger Ca$^{2+}$ currents ($I_{\text{Ca}}$) and the probability of Ca$^{2+}$ spark activation display a bell-shaped voltage dependence,36,37,40 whereas the properties of individual Ca$^{2+}$ sparks do not vary with the membrane voltage, or the duration and amplitude of $I_{\text{Ca}}$.35–37,40 This indicates that, once activated, a Ca$^{2+}$ spark evolves independently of its trigger. During full-fledged cardiac EC coupling, about 10$^4$ sparks are activated within a few tens of milliseconds in a single myocyte,33 summing into a global [Ca$^{2+}$] transient of $\approx$1 $\mu$mol/L.

Similar Ca$^{2+}$ sparks have also been recorded in intact cardiac trabeculae.41 In atrial myocytes, which lack transverse tubules (TT) and contain both peripheral junctional and central nonjunctional SR, spontaneous Ca$^{2+}$ sparks are greater and longer than the ventricular counterparts (300 000 Ca$^{2+}$ ions in 12 ms versus 100 000 Ca$^{2+}$ ions in 7 ms), with a high prevalence at the periphery.42,43 The action potential directly evokes subsurface Ca$^{2+}$ sparks in a stochastic fashion, which subsequently activate inwardly propagating waves of CICR.42 Rhythmic Ca$^{2+}$ sparks and compound Ca$^{2+}$ sparks are also found to regulate diastolic depolarization and thereby the pacemaker activity in sinoatrial nodal cells.44,45

High Ca$^{2+}$ microdomains, resulting from intracellular Ca$^{2+}$ release, are now found in many other cell types. In particular, Ca$^{2+}$ sparks are present in skeletal46,47 and smooth muscle cells48 containing different isoforms of RyRs. In addition to CICR, depolarization-induced Ca$^{2+}$ sparks in amphibian skeletal muscle fibers are generated by mechanical coupling between RyRs and DHPRs, the voltage sensors.46,47 IP$_3$ receptor (IP,R)–mediated “Ca$^{2+}$ puffs”49 or “Ca$^{2+}$ blips”50 have been shown to originate from discrete CRUs in Xenopus oocytes. Even in nonexcitable cells, local Ca$^{2+}$ release events with typical spark characteristics have been visualized and attributed to the activation of RyRs and/or IP$_3$R51,52. However, there is less information about the existence and role of Ca$^{2+}$ sparks in neurons. Although excessive “noise” or bright pixels have been detected by histogram analysis of neuronal Ca$^{2+}$ images, no discernible spark events were actually resolved.53 The elementary Ca$^{2+}$ release events in nerve growth factor–differentiated PC12 cells or cultured hippocampal neurons exhibit distinctly greater width ($\approx$4 $\mu$m) and duration ($\approx$400 ms),54 suggesting that they arise from spatially extended CRUs or represent compound sparks.

As a technical note, fluo-3 and its high-fluorescence derivative fluo-4 have thus far been the indicator of choice in spark experiments. This is because of their unique combination of superb signal-to-background contrast (200 times more brilliant on Ca$^{2+}$ association), visible light excitation, appropriate kinetics, and dissociation constant ($K_d$=0.4 $\mu$mol/L in physiological saline55, $\approx$1.1 $\mu$mol/L in skeletal muscle cells due to protein-binding of the indicator$^{60}$). However, neither indicator displays a significant shift in excitation or emission spectrum on Ca$^{2+}$ association, which precludes the use of ratiometric measurement and poses a problem in determination of the absolute [Ca$^{2+}$] involved. Wide ranges of image techniques have been applied to investigate Ca$^{2+}$ sparks in different cell types. These include single-photon and two-photon excitation confocal microscopy,57 and nonconfocal methods such as total internal reflection fluorescence microscopy (TIRFM)$^{58}$ and wide-field microscopy coupled with a low-noise CCD camera.59 In fact, cardiac Ca$^{2+}$ sparks are visible to the human eye with the aid of a conventional epifluorescence microscope (H.C., personal observation, 2003).

Subspark and Sparkless Ca$^{2+}$ Release Events

Interestingly, ectopic expression of cardiac60 or skeletal61 RyR in CHO cells is associated with a sparkless type of Ca$^{2+}$ release. In guinea pig ventricular myocytes, Lipp and Niggli first reported sparkless release in response to photolysis of caged Ca$^{2+}$.62 and later demonstrated subtler Ca$^{2+}$ release events, named “Ca$^{2+}$ quarks.”63 The sparkless appearance indicates that the elementary release events involve a quantity of Ca$^{2+}$ that is well beyond the detection limit.

Mammalian adult skeletal muscle fibers manifest a sparkless form of release under physiological conditions.64 The irony is that the same release machinery can also generate spark-featured release after chemical permeabilization of the surface membrane.65 In immature skeletal muscle cells from mice, EC coupling at spatially segregated sites66,67 displays a sparkless type of release, whereas discrete sparks occur at locations devoid of direct EC coupling.66 These observations led to the proposal that DHPRs function also to suppress the mechanism that activates discrete release events.54 Because
repolarization can abruptly terminate on-going Ca\(^{2+}\) sparks in amphibian skeletal muscle cells,\(^69\) a rapid “turn-on” and “turn-off” of RyRs by DHPRs could possibly explain the apparent lack of discrete Ca\(^{2+}\) sparks in the mammalian species.

**Morphometric Analysis of Microdomain Ca\(^{2+}\)**

Generic problems for fluorescence-based imaging of microdomain Ca\(^{2+}\) include (1) out-of-focus blurring, particularly when the placement of the Ca\(^{2+}\) sources in relation to the focal volume is uncertain, (2) optical blurring, as defined by the point spread function (PSF) of the imaging system, and (3) kinetics and diffusion of the indicator. In this section, we will use morphometric analysis of Ca\(^{2+}\) sparks as a showcase to discuss these issues in quantitative measurements of microdomain Ca\(^{2+}\).

Confocal sampling theory states that out-of-focus blurring will produce a reduced spark amplitude, broadened spatial spreading, and blunted kinetics with the degree of distortion depending on the relative positioning.\(^12,13\) Because there are more sparks at out-of-focus than in-focus places, it was predicted that the apparent spark amplitude always obeys a monotonically decaying distribution, regardless of the true spark amplitude.\(^69,70\) Experiments aided with an automated spark detection algorithm corroborated this prediction.\(^69,71\)

Several approaches have been developed to curtail or correct for the effects of out-of-focus blurring. In an effort to restore the true population statistics of spark amplitude, Izu et al\(^70\) and Rios et al\(^72\) have attempted to deconvolve the blurring from the apparent spark amplitude distribution. Some investigators have exploited spark repeats from hyperactive CRUs\(^73,74\) or repeatedly evoked Ca\(^{2+}\) sparks at fixed TT-SR junctions\(^38\) to analyze the variability of spark parameters. We have recently provided a solution to this problem by activating Ca\(^{2+}\) sparks from in-focus CRUs.\(^75,76,77\) Specifically, a low-resistance (20 to 50 MΩ) seal was formed by gently pressing a patch pipette against the surface membrane without disrupting the exquisite EC coupling machinery. A population of in-focus Ca\(^{2+}\) sparks (Figure 1B) can then be evoked by repeated patch depolarization. A more rigorous characterization of spark properties revealed that the true amplitude of Ca\(^{2+}\) sparks exhibits a broad, modal distribution,\(^75,76\) in contrast to the notion that Ca\(^{2+}\) sparks are all-or-none and stereotypical.\(^6,35,36\)

Because of the finite PSF of the optical system, even the in-focus Ca\(^{2+}\) spark is not blur-free. A typical PSF used for confocal Ca\(^{2+}\) spark recording is 0.3 to 0.4 μm (measured as the full width at half-maximum, FWHM) in the radial direction and 0.7 to 1.5 μm in the axial direction, the latter being comparable to the FWHM of a spark. The sharpest optical focus is attainable with TIRFM, which is about 200 nm into the cell. In future studies, digital deblurring in conjunction with ultrafast 3-dimensional (x-y-z) confocal imaging may further improve the measurement morphometrics of in-focus sparks.

Apart from the cellular determinants of local [Ca\(^{2+}\)] gradients discussed earlier, the fluorescence signal is further shaped up by the reaction kinetics and diffusion of the indicator.\(^12–14\) Thus, even the blur-free fluorescence signal differs markedly from the underlying Ca\(^{2+}\) signal. A direct conversion of the fluorescence signal to [Ca\(^{2+}\)] (assuming equilibrium between local Ca\(^{2+}\) and the indicator) would grossly underestimate [Ca\(^{2+}\)] at the origin, but slightly overestimate [Ca\(^{2+}\)] at the far site, while its time-to-peak tracks the duration of Ca\(^{2+}\) release.\(^13\) Finally, it should be cautioned that inclusion of the Ca\(^{2+}\) indicator disturbs the microscopic [Ca\(^{2+}\)] gradients per se; photochemical and metabolic products of the indicator may introduce additional complications to the [Ca\(^{2+}\)] measurement and cause significant damage to the cell.

**Cellular Organization of Ca\(^{2+}\) Microdomains**

The cellular organization of spark ignition sites has been investigated in ventricular myocytes by linescan imaging along the transverse and longitudinal axes of the cell.\(^32,78\) Fast 2-dimensional (x-y) imaging in the presence of excessive Ca\(^{2+}\) buffers,\(^79\) and 2-dimensional mapping of evoked Ca\(^{2+}\) sparks in high K\(^{+}\) depolarized myocytes (Figure 2). Simultaneous staining of the TT space\(^33,34\) or membrane\(^12,78\) has consistently shown that Ca\(^{2+}\) sparks are predominantly localized to the Z-line/TT regions of sarcomeres, with a spacing of 1.8 μm (a sarcomere length) in the longitudinal direction. Laterally, they are, on average, 0.8 μm apart\(^2\) (Figure 2).

The disposition of functional CRUs provides the basis for intrasarcemeric [Ca\(^{2+}\)] gradients and spatial inhomogeneities of [Ca\(^{2+}\)] transients seen during EC coupling.\(^33,79–81\)

The density of functional CRUs is estimated to be ≈1 CRU/μm\(^2\), or ≈10\(^4\) CRUs per myocyte. The estimated density of functional CRUs is thus in remarkable agreement with the data from thin-section electronic microscopic micrographs.\(^82\) Given that a cell contains about 10\(^8\) RyRs,\(^3\) this suggests an average grouping of ≈100 RyRs in a CRU. This estimate, however, differs considerably from the ultrastructural data (267 RyRs per CRU in rat ventricular myocytes,
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Figure 3. **Ca\textsuperscript{2+} spikes and the gain function of EC coupling.** Whole-cell patch clamp was established with the dialysis of 1 mmol/L Oregon Green 488 BAPTA 5N and 4 mmol/L EGTA. Ca\textsuperscript{2+} spikes were elicited at ~30 mV. Data are shown as surface plot and vertical dashed lines mark the beginning and end of the voltage pulse. B. Bell-shaped voltage dependence for SR Ca\textsuperscript{2+} release flux (J_{\text{SR}}) measured by spatially averaged Ca\textsuperscript{2+} spikes (open symbols) and I_{\text{Ca}} (solid symbols). C. Voltage dependence of the gain function (J_{\text{SR}}/I_{\text{Ca}}) (data from Cheng and Wang\textsuperscript{10} and Song et al\textsuperscript{81}).

“clamp” the bulk Ca\textsuperscript{2+}, suppress global [Ca\textsuperscript{2+}] transient, and cause a slow refilling of the SR.\textsuperscript{81,86,87}

In heart muscle cells, Ca\textsuperscript{2+} spikes have been used to track Ca\textsuperscript{2+} release flux during spontaneous Ca\textsuperscript{2+} sparks\textsuperscript{74} and TT-SR junction activation during full-fledged EC coupling (Figure 3).\textsuperscript{81,86,87} The TT-SR Ca\textsuperscript{2+} spike reflects a single or a compound Ca\textsuperscript{2+} spark, depending on the number of CRUs recruited. The magnitude of spatially averaged Ca\textsuperscript{2+} spikes or the SR release function (J_{\text{SR}}) exhibits a bell-shaped voltage dependence (Figure 3B), but the gain function, defined as J_{\text{SR}}/I_{\text{Ca}} is a monotonic decreasing function of voltage (Figure

assumed CRU geometry).\textsuperscript{82} This discrepancy may stem from the lack of knowledge of the exact shape of CRUs. Similarly, a 3-dimensional grid of CRUs spaced at ~2 \textmu m apart has been deduced from functional data and immunostaining of RyRs in atrial myocytes.\textsuperscript{42}

Thus, in cardiac myocytes as a model system, it is no longer considered adequate to depict cellular Ca\textsuperscript{2+} signaling as a common-pool system governed by deterministic laws. Rather, it is a discrete, stochastic system that encompasses tens of thousands of Ca\textsuperscript{2+} microdomains operating relatively independently. In this new paradigm, cellular Ca\textsuperscript{2+} signaling is orchestrated by these localized, short-lived Ca\textsuperscript{2+} microdomains. Hence, regulation of Ca\textsuperscript{2+} signaling can be achieved by varying the number of microdomains recruited (from 1 to 10\textsuperscript{s}) and by modulating the amount of Ca\textsuperscript{2+} released in individual microdomains through both global and local control mechanisms. Assuming each microdomain can exist in at least two distinct states (“on” and “off”), the number of patterned activation of Ca\textsuperscript{2+} microdomains in a cell would be astronomical (10\textsuperscript{3000}). Furthermore, some Ca\textsuperscript{2+} transducing molecules such as Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII)\textsuperscript{10} can retain a “memory” of the rate and duration of recent Ca\textsuperscript{2+} pulses, and render a frequency-encoding phosphorylation of downstream effectors (eg, phospholamban in heart cells),\textsuperscript{84} thereby enriching the versatility of spatiotemporal Ca\textsuperscript{2+} signaling.

### Ca\textsuperscript{2+} Spikes: Visualization of Local Ca\textsuperscript{2+} Fluxes

Injecting Ca\textsuperscript{2+} into a heavily buffered medium would produce a surge of free [Ca\textsuperscript{2+}], dubbed a “Ca\textsuperscript{2+} spike,” reflecting a kinetic imbalance between Ca\textsuperscript{2+} ions and the buffer.\textsuperscript{85} In the limit of a high concentration of Ca\textsuperscript{2+} buffer, the Ca\textsuperscript{2+} spike follows the waveform of Ca\textsuperscript{2+} injection flux.\textsuperscript{81} By exploiting this principle, we devised a method to visualize localized Ca\textsuperscript{2+} release flux or Ca\textsuperscript{2+} spikes (Figure 3).\textsuperscript{81} This involves an admixture of a fast, low-affinity Ca\textsuperscript{2+} indicator (eg, Oregon Green 488 BAPTA 5N and 4 mmol/L EGTA) and an excess of high affinity, but slow Ca\textsuperscript{2+} chelator (eg, EGTA, 5 mmol/L, [K\textsubscript{e}]=150 mmol/L at pH 7.2). As Ca\textsuperscript{2+} ions are emitted from an effective point source, the initial ratio of Ca\textsuperscript{2+} binding to the fluorescent indicator (F) and to EGTA is determined by k_{\text{on,F}}×(F)/k_{\text{on,EGTA}}×[EGTA]. Because k_{\text{on,F}} is typically about 100-fold faster than k_{\text{on,EGTA}}, EGTA should exert little effect on the indicator signal in the close vicinity of the channel pore. As Ca\textsuperscript{2+} ions diffuse outward, however, nearly all of them will quickly be captured by the nonfluorescent EGTA at excess. As a result, this rather unusual experimental setup helps to track the time course and pinpoint the origin of local Ca\textsuperscript{2+} flux.

For the same reasoning that EGTA does not suppress the local indicator signal, inclusion of EGTA at mmol/L concentrations should not significantly affect CICR between LCCs and RyRs\textsuperscript{81,86,87} nor the retrograde inactivation of LCCs by Ca\textsuperscript{2+} through RyRs,\textsuperscript{88} both occurring on the nanometer scale. Nevertheless, excessive Ca\textsuperscript{2+} buffering would hamper Ca\textsuperscript{2+} communication on the micrometer scale, such as CICR between adjacent CRUs. Additionally, high [EGTA] would...
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Peng et al 95 have developed an optical bilayer system that is recording of Ca$^{2+}$ capable of simultaneously visualizing single-channel Ca$^{2+}$ cation channels92 or stretch-activated cation channels in transients) arising from the openings of caffeine-sensitive RyRs underlies termination of cardiac Ca$^{2+}$ notion that a highly localized, use-dependent inactivation of variables that determine the space-time characteristics of microdomain Ca$^{2+}$, to calibrate the fractional Ca$^{2+}$ current through nonselective Ca$^{2+}$ channels (eg, RyRs), and to

**Optical Recordings of Ca$^{2+}$ Flux Through Single Ca$^{2+}$-Permeable Channels**

Ca$^{2+}$ ions passing through a single opening of a single Ca$^{2+}$-permeable channel create a Ca$^{2+}$ microdomain that is fundamental to intracellular Ca$^{2+}$ signaling. Although Ca$^{2+}$ sparks were initially thought to be a single-channel phenomenon, a consensus has evolved that most Ca$^{2+}$ sparks are of multi-RyR origin.6,7,10,15,77–79,101 Niggli and colleagues have attributed Ca$^{2+}$ quarks to single-RyR events62,63 and Parker and colleagues have attributed Ca$^{2+}$ blips to single-IP3,10 events; but experimental evidence thus far is equivocal as to the single-channel nature of the subtler release events they observed. Using confocal microscopy combined with the cell-attached patch-clamp technique, we provided an unequivocal demonstration of the feasibility of recording Ca$^{2+}$ sparklets, microscopic [Ca$^{2+}$] transients produced by single Ca$^{2+}$-permeable channel openings in intact cells.73 This was done for cardiac LCCs with the aid of the channel agonist FPL64176 to prolong the channel open duration (Figure 4).

Simultaneous recording of unitary Ca$^{2+}$ currents indicates that the total fluorescence of a Ca$^{2+}$ sparklet in the linescan image correlates linearly with the amount of Ca$^{2+}$ entry through the channel (Figure 4B).75 This indicates that Ca$^{2+}$ sparklet serves as a faithful optical readout of single-channel Ca$^{2+}$ flux.

Using a wide-field imaging system equipped with a high-speed, low-noise CCD camera, Zou et al have independently visualized Ca$^{2+}$ sparklets (single-channel Ca$^{2+}$ fluorescence transients) arising from the openings of caffeine-sensitive cation channels92 or stretch-activated cation channels in smooth muscle myocytes.93 Because a wide-field microscope collects light from both in-focus and out-of-focus planes, the 2-dimensional image it acquires is a spatial integration itself. While this may not be desirable for looking at localized fluorescence transient in its focal plane, it is valuable in determining the local Ca$^{2+}$ flux by “signal mass” (total fluorescence signal).95

Most recently, Demuro and Parker94 succeeded in confocal recording of Ca$^{2+}$ sparklets produced by individual N-type voltage-gated Ca$^{2+}$ channels expressed in Xenopus oocytes. Peng et al95 have developed an optical bilayer system that is capable of simultaneously visualizing single-channel Ca$^{2+}$ flux and recording unitary ionic current in planar lipid bilayers. Using this experimental system, they detected bi-

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**Figure 4.** Visualization of single-channel Ca$^{2+}$ sparklets. A, Ca$^{2+}$ sparklets (top) due to single LCC Ca$^{2+}$ current, $i_{Ca}$ (bottom). Cell-associated GΩ-seal patch clamp was established with the pipette containing 10 μmol/L FPL64176 and 20 mmol/L Ca$^{2+}$. SR Ca$^{2+}$ release was disabled by caffeine (10 mmol/L) and thapsigargin (10 μmol/L). B, Correlation between sparklet signal mass (II$\Delta F/F_0$dxdt) (in arbitrary unit) and the integral of the corresponding $Q_{Ca}$ (number of Ca$^{2+}$ ions) (data from Wang et al29). C, Ca$^{2+}$ sparklet evoked at −50 mV from a TT inside a rat ventricular myocyte. Whole-cell voltage-clamp configuration was established in the presence of 10 μmol/L FPL64176 and 10 mmol/L extracellular Ca$^{2+}$ and confocal plane was focused −5 μm into the cell. SR Ca$^{2+}$ release was inhibited as in A. D, Parallel optical recording of LCC Ca$^{2+}$ sparklets from deep inside a cell.
investigate possible coordinated operation of RyRs in a 2-dimensional array.\textsuperscript{96,97} By analysis of the quantal substructure found in the distribution of Ca\textsuperscript{2+} currents in a spark (\(I_{\text{spark}}\)), we have identified a subpopulation (12\%) of Ca\textsuperscript{2+} sparks that consist of a single quantum of 1.2 pA with a mean release duration of 16.7 ms.\textsuperscript{77} If a quantum reflects a single-RyR phenomenon, this represents the first measurement of unitary current and gating kinetics of RyR in intact cells.

Optical Single-Channel Recording: Advantages, Limitations, and Potentials

The introduction of the patch-clamp technique in early 1980s has revolutionized our understanding of ion channel function by allowing measurement of single-channel currents at unprecedented resolution.\textsuperscript{98} As illustrated earlier, modern optics, photonics, and the use of ion-selective indicators have extended the horizon by affording a new modality for monitoring single Ca\textsuperscript{2+} channel activity. Complementing the electrophysiological approaches, optical single-channel recording possesses unique advantages along with its own set of limitations.

First, optical single-channel recording is penetrative. It is widely applicable to channels inaccessible to electrophysiological means, such as those on intracellular organelles (Figure 1) or invaginations (TTs) of the plasma membrane (Figures 4C and 4D), or some distance into a tissue or organ preparation. Second, it is inherently nondestructive. No mechanical stress is exerted to disrupt the integrity of membrane features (eg, cavoelli), cytoskeleton, channel array assembly, and macromolecular channel complexes. However, the inclusion of exogenous Ca\textsuperscript{2+} indicator and light radiation may perturb Ca\textsuperscript{2+} signaling processes and even damage the cell. Third, the optical channel recording is capable of parallel readout, while retaining resolution for individual events (Figure 4D). For instance, a longitudinal linescan of a cardiac cell can survey \(\approx10^4\) RyRs at the same time.\textsuperscript{6} This is useful to catch rare, serendipitous, yet biologically relevant events (eg, cyclic activation of RyRs at fixed sites\textsuperscript{45,73,74}) and to map regional differences of channel behavior.\textsuperscript{99} Last, but not least, the ion selectivity of the indicator offers the ability to discriminate among ion species through the same channels. This feature has been exploited to measure the fractional Ca\textsuperscript{2+} flux through nonsellective Ca\textsuperscript{2+}-permeable channels, at the whole-cell\textsuperscript{99} or single-channel level.\textsuperscript{93}

Ca\textsuperscript{2+} sparklets produced by a known Ca\textsuperscript{2+} current can be used as a natural “yardstick” to calibrate any unknown local Ca\textsuperscript{2+} flux, such as \(I_{\text{spark}}\). Sparklet-based measurements show an average \(I_{\text{spark}}\) of 2 to 3 pA.\textsuperscript{75,76} This value falls to the lower bound of the \(I_{\text{spark}}\) (1.5 to 20 pA) derived from forward modeling,\textsuperscript{12-15} model-based fitting,\textsuperscript{57} or backward-calculation of the release flux from Ca\textsuperscript{2+} sparks.\textsuperscript{100} Because Ca\textsuperscript{2+} sparklets and Ca\textsuperscript{2+} sparks share common microenvironments in terms of Ca\textsuperscript{2+} buffering, indicator binding, and Ca\textsuperscript{2+} and indicator diffusion, the optical calibration relies on no assumption other than a linear extrapolation.

A fundamental distinction between the optical and electrophysiological approaches exists in respect to the physical principles used. A single Ca\textsuperscript{2+} ion moving through a channel contributes two positive electron charges to the electrical current, whereas a Ca\textsuperscript{2+}-bound fluorochrome can emit 10\(^4\) photons per millisecond with saturating excitation.\textsuperscript{55} This represents a staggering power of amplification in terms of the number of information carriers involved. Although only a small fraction of these photons can be harvested and contribute to the output signal, future innovations might bring out the vast potential inherent in the optical single-channel recording technique.

At present, a major limiting factor for optical Ca\textsuperscript{2+} channel recording is insufficient temporal resolution, which is due to the reaction and diffusion kinetics of microdomain Ca\textsuperscript{2+} and the indicator as well as the photon collection efficiency. For confocal detection of Ca\textsuperscript{2+} sparklets, the smallest quantity of Ca\textsuperscript{2+} resolved in an in-focus single-channel event was about 8000 Ca\textsuperscript{2+} ions,\textsuperscript{75} corresponding to a unitary current of 1 pA lasting 2.5 ms (Figures 4A and 4B). The temporal resolution achieved in another study was at best about 10 ms.\textsuperscript{94} The limited temporal resolution has hampered efforts to resolve Ca\textsuperscript{2+} sparklets under physiological conditions where LCC current is \(\approx0.12\) pA at 0 mV (2 mmol/L Ca\textsuperscript{2+} as the charge carrier)\textsuperscript{101} and lasts \(\approx0.3\) ms,\textsuperscript{102} carrying a packet of \(\approx110\) Ca\textsuperscript{2+} ions. Future technical innovations, particularly in the area of information-carrier amplification, are needed to improve the temporal resolution of optical single-channel recording in situ and in vitro.

Visualization of Intermolecular Ca\textsuperscript{2+} Signaling

In heart cells, LCCs and RyRs colocalize to dyadic junctions where the surface membrane or TT comes into direct apposition to the SR membrane at a 12-nm distance. Within the nanoscale junctional cleft, stochastic gating of single LCCs is expected to deliver the trigger Ca\textsuperscript{2+} pulses to activate Ca\textsuperscript{2+}-gated RyRs. Two groups have independently suggested that single LCC excitation is sufficient to trigger a Ca\textsuperscript{2+} spark. Under conditions where most LCCs are inhibited (for resolution of solitary sparks), Lopez-Lopez et al\textsuperscript{14} demonstrated kinetic similarities for \(I_{\text{Ca}}\) and spark activation. We have noticed that the voltage dependence of Ca\textsuperscript{2+} spark activation (\(P_s\)) is proportional to LCC activation (\(P_{\text{LCC}}\)) at near-threshold voltages (from -60 to -40 mV), displaying an \(e\)-fold increment per 7-mV depolarization.\textsuperscript{35,37} Both lines of evidence suggest that spark activation does not require cooperative interaction among LCCs; otherwise, \(P_s\) would be expected to be a power function of \(P_{\text{LCC}}\), or \(P_s\approx P_{\text{LCC}}^{x>1}\), with \(x>1\).

Attempts have been made to demonstrate that a single LCC can trigger a spark.\textsuperscript{75,77,103} By using simultaneous optical and electrophysiological single-channel recording techniques, we have directly visualized the triggering of a Ca\textsuperscript{2+} spark by at the opening of a single LCC (Figure 5).\textsuperscript{75} This represents a real-time demonstration of nanoscale crosstalk between two sets of molecules functioning in cells at the molecular level of resolution.

Simultaneous visualization of LCC Ca\textsuperscript{2+} sparklets and RyR Ca\textsuperscript{2+} sparks (Figure 5B) permitted us to determine the kinetics, fidelity, and stoichiometry of the intermolecular coupling. In contrast to the robust EC coupling at the cellular level, Ca\textsuperscript{2+} sparklets do not always trigger a spark. The latency from the onset of the sparklet to the ignition of the
sparks is well described by a single exponential function with a time constant of 6.7 ms. This indicates that the LCC-to-RyR coupling is governed by first order kinetics.

Based on these results and macroscopic properties of EC coupling, we have estimated that, on average, one out of \( \approx 50 \) LCC openings at 0 mV triggers a \( \text{Ca}^{2+} \) spark in the absence of the LCC channel agonist FPL64176. \(^{10,104}\) At high voltages and increased LCC open probability, there could be more than one LCC opening simultaneously in a junctional cleft. \( \text{Ca}^{2+} \) spark activation under these conditions may involve two or more LCCs, as has been suggested recently. \(^{105}\)

Recent work has also begun to explore crosstalk of arrayed RyRs within a CRU in cardiac \(^{76,77}\) and skeletal muscle cells. \(^{91}\) In a model recently advanced by us, \(^{77}\) cardiac spark genesis involves variable cohorts of RyRs ranging from one to eight, as evidenced by the varying number of quanta in \( I_{\text{spark}} \). A negative feedback or autoregulation mechanism results in an inverse relationship between \( \text{Ca}^{2+} \) release duration and the number of RyRs activated. \(^{77}\) A third well-characterized case of intermolecular \( \text{Ca}^{2+} \) signaling involves RyRs in the SR and large-conductance \( \text{Ca}^{2+} \)-sensitive K\(^+\) (BK Ca\(_{\text{Ca}}\)) channels in the plasma membrane of smooth muscle myocytes. \(^{48,59,106}\) This will be briefly addressed later.

### \( \text{Ca}^{2+} \) in Subcellular Compartments

\( \text{Ca}^{2+} \) transients immediately beneath the cell membrane are critically involved in physiological processes such as EC coupling, hormone secretion, and neurotransmitter release, which are thought to be driven by \( \text{Ca}^{2+} \) concentration 10 to 100 times higher than those measured in the bulk cytosol. However, near-membrane \( \text{Ca}^{2+} \) signal is usually obscured by signals from the bulk cytosol when diffusive water-soluble indicators are used (except for under conditions of \( \text{Ca}^{2+} \) spike measurement). Llinas et al.\(^{107}\) used the low-affinity, highly nonlinear chemiluminescent \( \text{Ca}^{2+} \) indicator, \( \text{n}-\text{aequorin-J} \), to demonstrate the existence of high subsurface \( [\text{Ca}^{2+}] \) domains during neuronal transmitter release. Etter et al have made several attempts to resolve near-membrane \( [\text{Ca}^{2+}] \) using membrane-associated fluorescent \( \text{Ca}^{2+} \) indicators, \( \text{C}\(_{\text{ae}}\)-fura-2 \( (K_d=150 \text{ nmol/L}) \))\(^{108}\) and FFP18 \( (K_d \approx 400 \text{ nmol/L}, \text{depending on indicator concentration used}) \))\(^{109}\) in smooth muscle cells. They found that \( [\text{Ca}^{2+}] \) transients exhibit a greater rising rate and briefer duration in the submembrane layer than in the bulk cytosol as expected from the membrane association of \( \text{Ca}^{2+} \) sparks and sparklets.

As discussed earlier, microscopic \([\text{Ca}^{2+}]\) transients might also exist in membrane-bound subcellular organelles. Recently, Shannon et al.\(^{110}\) developed a technique to image SR luminal \([\text{Ca}^{2+}]\) in rabbit cardiac myocytes (Figure 6) that involves \( \text{AM-loading of the SR with a low-affinity indicator, fluo-5N} \), by an empirical method. During an action potential–triggered release, there was about 50% global depletion of the SR \( \text{Ca}^{2+} \). They named the negative SR \( \text{Ca}^{2+} \) depletion transients “\( \text{Ca}^{2+} \) scraps.”\(^{110}\) Importantly, there is no detectable intraluminal \( [\text{Ca}^{2+}] \) gradient between the junctional SR at the TT region (the release site) and the longitudinal SR (the uptake site).\(^{110}\) This suggests a rapid translocation of \( \text{Ca}^{2+} \) inside the SR network and challenges local \( \text{Ca}^{2+} \) depletion as the mechanism underlying the termination of \( \text{Ca}^{2+} \) sparks.\(^{111,112}\) Direct visualization of local SR \( \text{Ca}^{2+} \) during a \( \text{Ca}^{2+} \) spark should be informative in elucidating \( \text{Ca}^{2+} \) spark termination mechanism as well as SR luminal \( \text{Ca}^{2+} \) regulation.

Albeit inconsequential to beat-to-beat EC coupling, \( \text{Ca}^{2+} \) trafficking across the mitochondrial membrane is crucial to noncontractile cardiac function, such as energy metabolism and \( \text{Ca}^{2+} \)-mediated cell death. Using rhod-2AM loading of mitochondria, Pacher et al.\(^{113}\) demonstrated single mitochondria \([\text{Ca}^{2+}] \) transients, dubbed “\( \text{Ca}^{2+} \) marks,” in cardiac myotubes differentiated from H9c2 cells. \( \text{Ca}^{2+} \) marks are thought to be triggered by RyR \( \text{Ca}^{2+} \) sparks in close proximity to the mitochondria, but they usually outlast the trigger sparks by hundreds of milliseconds. The relevance and significance of \( \text{Ca}^{2+} \) marks, and the exact relationship between \( \text{Ca}^{2+} \) sparks and \( \text{Ca}^{2+} \) marks, remain to be established in native cardiac myocytes.
Physiological Significance of Microdomain Ca$^{2+}$

As mentioned earlier, the demonstration of dynamic microdomain Ca$^{2+}$ in living cells has reshaped our understanding of cellular Ca$^{2+}$ signaling. In this section, we would like to present several prime examples of this to illustrate general Ca$^{2+}$ signaling principles revealed by imaging microdomain Ca$^{2+}$.

CICR Paradox

CICR as the control mechanism of SR Ca$^{2+}$ release was once thought to be paradoxical. Because CICR is intrinsically a positive feedback mechanism, it is expected to operate in an all-or-none fashion, unless the amplification gain is sufficiently low.114 In contrast, cardiac EC coupling is characterized by both high-gain amplification (≈10 at 0 mV) (Figure 3C)81,89 and a smoothly-graded response to the trigger $I_{Ca}$ (Figure 3B).81,89,115–117 To solve this CICR paradox, early experimental118 and theoretical work119 challenged the wisdom of the “common pool” model and invoked the “local control theory” of CICR. This theory consists of four postulates, each of which has now been validated experimentally.

1. RyRs in situ are relatively insensitive to physiological levels of global Ca$^{2+}$. The rate of occurrence of spontaneous Ca$^{2+}$ sparks is about 100 sparks per cell per second.6 Provided that 10$^{6}$ RyRs are present in a single myocyte,5 this translates into an opening rate of only 10$^{-4}$ s$^{-1}$ for RyR at the resting [Ca$^{2+}$]$_{i}$ of ≈100 nmol/L. At the cellular level, a sudden uniform increase in cytosolic [Ca$^{2+}$] produced by photolysis has also shown global CICR is intrinsically a low-gain amplification system.118

2. Yet, RyRs are effectively activated by local trigger Ca$^{2+}$. Indeed, individual Ca$^{2+}$ sparks can be evoked by LCC Ca$^{2+}$ sparklets.75–77 The peak rate of LCC-triggered spark activation was estimated to be on the order of 10$^{6}$ sparks per cell per second.33 That is, cardiac CICR manifests both high-gain (when triggered by local high [Ca$^{2+}$]$_{i}$) and low-gain behavior (when triggered by global low [Ca$^{2+}$]). Additionally, owing to channel “adaptation”119 or inactivation,88 RyR is highly responsive to pulse rather than sustained Ca$^{2+}$ signals.119,120 The initial responsiveness of RyRs strongly depends on the [Ca$^{2+}$]$_{i}$ raised to the power of $n$ or higher.37,120 These provide critical mechanisms for a CRU to discriminate between local trigger Ca$^{2+}$ pulses and the ambient Ca$^{2+}$. (3) There is little or no communication between discrete CRUs under physiological conditions. The discreteness of Ca$^{2+}$ sparks confirms that SR Ca$^{2+}$ release does not always initiate spatially regenerative CICR. Cellular and molecular factors that contribute to the uncoupling between CRUs include spatial segregation, steep [Ca$^{2+}$] gradients at a point source, and strong Ca$^{2+}$ buffering of the cytoplasm. (4) There is a robust mechanism terminating CICR within a CRU. The brevity of Ca$^{2+}$ sparks and the negative regulation of spark rise time by spark Ca$^{2+}$ release flux86,77 are suggestive of a strong negative feedback or autoregulation in a CRU. However, the exact nature of spark termination mechanism remains elusive86 and merits future investigation.

Opposing Roles of Ca$^{2+}$ in Regulation of Smooth Muscle Contraction

In arterial smooth muscle myocytes, an increase in global [Ca$^{2+}$]$_{i}$ is expected to initiate muscle contraction, as is the case in all types of muscles. Paradoxically, inhibition of Ca$^{2+}$ sparks causes vessel constriction.46 This is because subsurface Ca$^{2+}$ sparks are associated with hyperpolarizing spontaneous transient outward currents (STOCs)106 due to activation of clusters of BK$_{Ca}$ channels (Figure 7).48 The resultant membrane hyperpolarization shuts off tonic Ca$^{2+}$ entry through the voltage-operated LCCs. Therefore, the net result of the spark-STOC coupling is a reduction in global [Ca$^{2+}$] and vessel relaxation.

Local Activation of High-Threshold Ca$^{2+}$ Signaling

It is now appreciated that cardiac RyRs and smooth muscle BK$_{Ca}$ channels are intrinsically insensitive to the levels of [Ca$^{2+}$] attainable in the bulk cytosol, as are the processes of hormone secretion and neurotransmitter release. They would remain quiescent if they were not localized to high [Ca$^{2+}$] microdomains. To this end, the aforementioned 3-dimensional grid of CRUs and near-membrane high [Ca$^{2+}$] compartments would serve as the primary system for high-threshold Ca$^{2+}$ signal transduction.

Perspectives

In spite of the many advances in microdomain Ca$^{2+}$ imaging, there are new emerging examples of physiologically relevant Ca$^{2+}$ signaling mechanisms concealed in nanodomains (1 to 100 nanometers). In this regard, a fundamental limitation of the optical approach is its spatial resolution, which is ≈200 nm at best (as in TIFRM). Visualization of nanodomain Ca$^{2+}$ would require supraoptical resolution, which demands new and innovative approaches. A possible strategy might involve tethering or docking Ca$^{2+}$ indicators to molecularly defined locations through molecular and genetic manipulations.
It is predictable that next generation of Ca$^{2+}$ indicators and imaging methods will be called on to perfect the science and technology of cellular Ca$^{2+}$ imaging. Likewise, new areas of cellular Ca$^{2+}$ signaling will be vigorously explored in the near future. In particular, the targeted expression of protein-based Ca$^{2+}$ indicators to subcellular compartments,\textsuperscript{1,2,12} in conjunction with time-lapsed Ca$^{2+}$ technology of cellular Ca$^{2+}$ imaging, holds promise for uncovering new dimensions of the space-time architecture of cellular Ca$^{2+}$ dynamics. Moreover, these approaches should be useful in elucidating roles of Ca$^{2+}$ in long-term regulation of noncontractile cellular functions (eg, myocyte differentiation, growth, hypertrophy, migration, and apoptosis). We eagerly await the grander horizons that lie ahead.

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