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Ca^{2+} in the Cleft: Fast and Fluorescent

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Calcium ion (Ca^{2+}) is a universal second messenger that governs a vast array of biological phenomena, including muscle contraction, neuronal transmission, fertilization, aging, cell death and hormone secretion[1]. Perturbations of intracellular Ca^{2+} signaling underlie a host of pathologic states, including the myocardial diseases of ischemia/reperfusion injury, arrhythmia, myocyte hypertrophy, and heart failure[2].

Despite the pivotal role of Ca^{2+} homeostasis in myocyte physiology and disease, large gaps exist in our understanding of its compartmentalization within subcellular microdomains and its trafficking among them. A vast literature has glossed over the finely tuned handling of intracellular Ca^{2+}, its sites of storage, release, action, and reuptake, focusing instead on crude measures of bulk Ca^{2+} concentration. In so doing, the intricacies of spatiotemporal handling of Ca^{2+} – occurring at the scales of milliseconds and nanometers – are blurred, and our understanding is incomplete.

Appreciation of the pivotal role of Ca^{2+} in heart function dates to the late 19th century, when Sidney Ringer discovered that this cation is absolutely required for cardiac mechanical function[3]. Cardiac contraction is triggered by influx of a small amount of Ca^{2+} through voltage-gated L-type Ca^{2+} channels (LTCC) embedded in the cell surface membrane. This Ca^{2+} influx, in turn, triggers release of much larger amounts of Ca^{2+} from sarcoplasmic reticulum (SR) stores through ryanodine receptors (RyRs), a process termed Ca^{2+}-induced Ca^{2+} release (CICR). Thus, LTCC Ca^{2+} influx gates CICR; in the converse sense, Ca^{2+} released by CICR feeds back to control LTCC influx[4]. The end result is an elaborate, finely tuned, and self-regulated cascade of events that controls every heart beat.

This intricate dynamics of this process occurs within, and is facilitated by, the microarchitecture of the junctional cleft, the region within the myocyte separating the SR and intramyocyte invaginations of the cell membrane (T-tubules)[5]. This zone, also termed the dyad, represents a volume of ~10^{-3} fL in cardiac muscle[6], with ~12 nm separating the LTCC within the T-tubule from the RyR where SR Ca^{2+} release takes place[7].

Under steady-state conditions, Ca^{2+} entering through the LTCC is extruded back out of the cell by the Na^{+}/Ca^{2+} exchanger[8]. SR Ca^{2+} stores are replenished by the SERCA pump. Ca^{2+}

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concentrations in cellular microdomains, and their time-dependent changes, are also governed by Ca^{2+} buffering proteins, including calsequestrin and S100A1, and Ca^{2+} storage and release in mitochondria.

As complex as this picture is already, evidence is emerging that dyads differ in their structure and molecular architecture[9]. In a variety of heart diseases, T-tubule and dyadic junction remodeling are seen, which very likely participates in disease pathogenesis and distorted Ca^{2+} release[7]. For all these reasons, it is of obvious importance to glean a more sophisticated understanding of Ca^{2+} handling within the junctional cleft, ideally with the spatial and temporal resolution required by the underlying biology.

Ca^{2+} sensors

Using Ca^{2+}-sensitive bioluminescent probes and fluorescent indicators, whole cell Ca^{2+} concentration ($[Ca^{2+}]_i$) during both systole and diastole have been studied extensively. The first experiments visualizing intracellular Ca^{2+} transients were reported in frog heart[10] and canine Purkinje fibers[11] using the Ca^{2+}-sensitive bioluminescent protein aequorin. As aequorin is membrane impermeant, these and subsequent experiments were performed by injecting protein purified from the jellyfish *Aequorea* directly into cells. The associated technical challenges, coupled with modest signal-to-noise ratio afforded by the sensor, limited the utility of this approach.

Next, development of small molecule chemical fluorescence indicators with high Ca^{2+} affinity and fast kinetics, such as Fura-2[12], as well as use of acetoxymethylated esters for non-invasive cell loading, were breakthroughs that expanded the feasibility of Ca^{2+} imaging in single cardiomyocytes[13]. Later, the advent of confocal microscopy and fast, high-contrast fluorescein- and rhodamine-based Ca^{2+} indicators, such as Fluo-3, led to characterization of Ca^{2+} sparks, the result of single-dyad Ca^{2+} events[14].

Whereas development of these diffusible indicators greatly facilitated our understanding of cardiac EC coupling, they afforded neither the submicron spatial resolution nor the rapid on- and off-kinetics required for a complete understanding of this intricate Ca^{2+} biology. It is estimated that Ca^{2+} ions diffuse within myocytes at a rate of ~100 μm/s[15] and typically traverse a distance of ~1.8 μm[16]. Thus, conventional fluorescent Ca^{2+} indicators substantially underestimate local peak Ca^{2+} levels and report a low-resolution profile of local Ca^{2+} gradients[17].

The next leap in Ca^{2+} imaging technology was introduction of genetically encoded Ca^{2+} indicators (GECIs). GECIs offer advantages not achievable with conventional chemical Ca^{2+} probes, including precise targeting within cellular micro(nano)-compartments, prolonged imaging over days and months, and simultaneous multicolor Ca^{2+} imaging[18]. Among the most popular protein calcium sensors are the GCaMPs, which are variants of enhanced green fluorescent protein (cpEGFP) coupled to a C-terminal Ca^{2+} sensor (calmodulin) and a calmodulin-binding M13 peptide at the N terminus[19]. Upon Ca^{2+} binding, the calmodulin moiety interacts with the M13 peptide, triggeringstructural reorganization in proximity to the cpEGFP, deprotonation of the chromophore, and

increased fluorescence[20]. GCaMPs are rapidly emerging as valuable tools for *in situ* monitoring of Ca^{2+} activity in neurons[21] and heart[22].

In this issue of the *journal*, Despa et al[23] describe two novel Ca^{2+} sensors specifically targeted to the junctional cleft. One harbors GCaMP2.2, a T203V mutation of GCaMP2 with intensified brightness and dynamic range[21]. The second, GCaMP2.2$_{Low}$, is a variant with reduced Ca^{2+} affinity, harboring a D133E mutation. Each construct was targeted to the dyadic cleft by means of covalent coupling to the N-terminus of FKBP12.6, a protein which binds with high affinity and selectivity to RyRs. Both fusion proteins retained high affinity for RyRs (K_d ~ 15-45 nmol/L), albeit somewhat lower than native FKBP12.6 (1 nmol/L)[24]. A striated pattern of fluorescence, competed away by FKBP12.6, was observed in cultured adult rat ventricular cardiomyocytes, suggesting that both FKBP12.6-tagged sensors target selectively to the junctional cleft.

What have these new probes taught us?

GCaMP2.2$_{Low}$ manifests baseline fluorescence and a dynamic range similar to GCaMP2.2, but its affinity for Ca^{2+} is 10-fold lower (K_d 5 μmol/L vs. 450 nmol/L). These investigators capitalized on these differences to quantify the high [Ca^{2+}] expected in the junctional cleft. Prior to this work, estimates of Ca^{2+} transient amplitudes near Ca^{2+} release sites ranged between 10 μM[8] to 100 μM[25], and peak amplitude was estimated to occur 5 10 ms after depolarization, with recovery occurring within 15-50 ms[8, 26]. In contrast, the global Ca^{2+} transient within the cytoplasm is many-fold lower, reaching a maximum of ~1 μM within ~70-100 ms[8]. Thus, GCaMP2.2$_{Low}$, with its low Ca^{2+} affinity, could provide reliable measures of Ca^{2+} transients within the junctional cleft, whereas GCaMP2.2, with its high Ca^{2+} affinity, is optimal for measuring diastolic $[Ca^{2+}]_I$.

With these newly engineered probes in hand, these investigators first calibrated the targeted and untargeted sensors in the myocyte environment, which reduced affinities by ~2-fold. However, FKBP12.6-targeting did not alter the Ca^{2+} affinity of either. Next, they set out to measure Ca^{2+} dynamics, reporting that $[Ca^{2+}]_{Cleft}$ reaches higher levels (1.3 μM) with faster kinetics than global $[Ca^{2+}]_{Bulk}$ (0.5 μM at peak). However, estimates of peak $[Ca^{2+}]_{Cleft}$ reported with this sensor are much smaller than previous estimates, and the time to $[Ca^{2+}]_{Cleft}$ peak (46 ms) is also much slower (5 10 ms). GCaMPs manifest significantly slower response kinetics (typically τ_{on} 20 ms-1.4 s and τ_{off} 0.4-5 s) compared with synthetic BAPTA-based dyes (e.g. Oregon Green 488 BAPTA 5N, τ_{on} <1 ms)[27]. As acknowledged by the authors, the very rapid rises and falls in $[Ca^{2+}]_{Cleft}$ almost certainly exceed the kinetics of the GCaMP sensor. In this respect, it is highly likely that the low Ca^{2+} affinity, cleft-targeted GCaMP2.2$_{Low}$ significantly under-estimated peak Ca^{2+} transients.

That being said, these novel sensors are ideally suited to measuring diastolic $[Ca^{2+}]_{Cleft}$. As Ca^{2+} leak from the SR has been implicated in a variety of disorders, including heart failure[28], such measures are of great interest. To address this, the authors compared the signal emitted by GCaMP2.2 ($[Ca^{2+}]_{Bulk}$) with that emitted by GCaMP2.2-FKBP12.6 ($[Ca^{2+}]_{Cleft}$), noting that $[Ca^{2+}]_{Cleft}$ was nearly 2-fold higher than $[Ca^{2+}]_{Bulk}$ (194 μM versus 100 μM). Next, by selectively interrupting Ca^{2+} flux through LTCC versus RyRs, the

authors went on to provide evidence that this resting diastolic gradient of Ca^{2+} stems from RyR leak, rather than sarcolemmalCa^{2+} flux.

The future is fast and bright

Whereas these ingenious, cleft-targeted probes have provided novel insights already, challenges remain, especially given their slow kinetics. Recently, a new generation of fast GCaMPs was introduced[27]. One of them, GCaMP6f, is reported to have fast kinetics (the fastest among all currently known GCaMPs), high affinity, and robust dynamic range; it was successfully implemented to measure single neuron synapse events[29]. Shang et al[30] fused GCaMP6f with junctin or triadin and reported high resolution images of junctional Ca^{2+} at individual dyads, so-called "Ca^{2+} nanosparks". Nevertheless, GCaMP6f is still not fast enough to accurately track the extremely rapidly rising and fast-decaying Ca^{2+} gradient in the junctional cleft[31].

Whereas GCaMP2.2$_{Low}$ – FKBP12.6 may not provide accurate measures of junctional cleft Ca^{2+} transients during excitation, GCaMP2.2 – FKBP12.6 is an ideal sensor to measure $[Ca^{2+}]_{Cleft}$ during diastole. Because heart failure and cardiac arrhythmias are often associated with dyssynchronous Ca^{2+} leak in diastole, this probe is likely to prove useful in dissecting underlying mechanisms. Also, high diastolic $[Ca^{2+}]_{Cleft}$ may play an important role beyond governing E-C coupling by regulating local signaling cascades (e.g. calcineurin or CaMKII)[32]. As a result, these clever new tools are likely to be informative in multiple domains of cardiovascular biology.

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