New Methods in Cardiovascular Biology

Junctional Cleft \([Ca^{2+}]_c\) Measurements Using Novel Cleft-Targeted \(Ca^{2+}\) Sensors

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Rationale: Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) is regulated and signals differently in various subcellular microdomains, which greatly enhances its second messenger versatility. In the heart, sarcoplasmic reticulum Ca\(^{2+}\) release and signaling are controlled by local [Ca\(^{2+}\)] in the junctional cleft ([Ca\(^{2+}\)]\(_c\)), the small space between sarcolemma and junctional sarcoplasmic reticulum. However, methods to measure [Ca\(^{2+}\)]\(_c\) directly are needed.

Objective: To construct novel sensors that allow direct measurement of [Ca\(^{2+}\)]\(_c\).

Methods and Results: We constructed cleft-targeted [Ca\(^{2+}\)] sensors by fusing Ca\(^{2+}\)-sensor GCaMP2.2 and a new lower Ca\(^{2+}\)-affinity variant GCaMP2.2Low to FKBP12.6, which binds with high affinity and selectivity to ryanodine receptors. The fluorescence pattern, affinity for ryanodine receptors, and competition by untagged FKBP12.6 demonstrated that FKBP12.6-tagged sensors are positioned to measure local [Ca\(^{2+}\)]\(_c\) in adult rat myocytes. Using GCaMP2.2Low-FKBP12.6, we showed that [Ca\(^{2+}\)]\(_c\) reaches higher levels with faster kinetics than global [Ca\(^{2+}\)], during excitation–contraction coupling. Diastolic sarcoplasmic reticulum Ca\(^{2+}\) leak or sarclemmal Ca\(^{2+}\) entry may raise local [Ca\(^{2+}\)]\(_c\) above bulk cytosolic [Ca\(^{2+}\)], ([Ca\(^{2+}\)]\(_b\)), an effect that may contribute to arrhythmias and even transcriptional regulation. We measured this diastolic standing [Ca\(^{2+}\)]\(_c\)-[Ca\(^{2+}\)]\(_b\) gradient with GCaMP2.2-FKBP12.6 versus GCaMP2.2, using [Ca\(^{2+}\)] measured without gradients as a reference point. This diastolic difference ([Ca\(^{2+}\)]\(_c\)=194 nmol/L versus [Ca\(^{2+}\)]\(_b\)=100 nmol/L) is dictated mainly by the sarcoplasmic reticulum Ca\(^{2+}\) leak rather than sarclemmal Ca\(^{2+}\) flux.

Conclusions: We have developed junctional cleft-targeted sensors to measure [Ca\(^{2+}\)]\(_c\) versus [Ca\(^{2+}\)]\(_b\) and demonstrated dynamic differences during electric excitation and a standing diastolic [Ca\(^{2+}\)] gradient, which could influence local Ca\(^{2+}\)-dependent signaling within the junctional cleft. (Circ Res. 2014;115:339-347.)

Key Words: calcium signaling • myocytes, cardiac • sarcoplasmic reticulum

Ca\(^{2+}\) is a universal second messenger involved in activation/regulation of cellular processes as diverse as neurotransmitter release, muscle contraction, metabolism, hypertrophic signaling, and cell death. To fulfill such diverse roles and yet be specific enough to trigger distinct responses, information is encoded in the amplitude, temporal properties, and subcellular localization of Ca\(^{2+}\) signals. It is now largely recognized that Ca\(^{2+}\) is regulated and signals differently in various subcellular microdomains.\(^1\)–\(^3\) For example, GABA (γ-aminobutyric acid receptors) inhibition in hippocampal neurons results in spatially confined inhibition of Ca\(^{2+}\) transients shortly after a back-propagating action potential, which may play a key role in regulation of synaptic plasticity.\(^4\)

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In the heart, Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) is a key event for excitation–contraction coupling (ECC), ventricular arrhythmias, mitochondrial function, and nuclear transcription.\(^5\) Cardiac SR Ca\(^{2+}\) release is controlled by the local [Ca\(^{2+}\)] in the junctional cleft ([Ca\(^{2+}\)]\(_c\)), the small, restricted space between the apposing sarcolemma (mostly T-tubules) and junctional SR membrane. Voltage-gated L-type Ca\(^{2+}\) channels (LTCC) and Ca\(^{2+}\)-activated Ca\(^{2+}\) channels (or ryanodine receptors [RyRs]) are located at these junctions in the sarcolemma and SR membrane, respectively, and are essential in this local control.\(^6\)–\(^8\) During electric excitation, [Ca\(^{2+}\)]\(_c\) is expected to rise higher and faster than in the bulk cytosol.\(^9\)–\(^12\) Through Ca\(^{2+}\)-dependent inactivation, [Ca\(^{2+}\)]\(_c\) feeds back on LTCC and thus plays an important role in shaping the action potential waveform. Several studies\(^12\)–\(^17\) suggest that the Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX), the main route of Ca\(^{2+}\) extrusion in cardiac myocytes, also has access to the cleft and influences [Ca\(^{2+}\)]\(_c\) during both diastole and ECC. In diastole, NCX may
Nonstandard Abbreviations and Acronyms

- [Ca\textsuperscript{2+}]\textsubscript{i}: intracellular Ca\textsuperscript{2+} concentration
- [Ca\textsuperscript{2+}]\textsubscript{junct}: local [Ca\textsuperscript{2+}] in the junctional cleft
- [Ca\textsuperscript{2+}]\textsubscript{bulk}: [Ca\textsuperscript{2+}] in the bulk cytosol
- ECC: excitation-contraction coupling
- LTCC: L-type Ca\textsuperscript{2+} channels
- NCX: Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger
- RyR: ryanodine receptors
- SR: sarcoplasmic reticulum

help to keep [Ca\textsuperscript{2+}]\textsubscript{junct} low and limit spontaneous RyR activation. Indeed, during stochastic RyR opening events, Ca\textsuperscript{2+} removal by NCX may limit local Ca\textsuperscript{2+} release within a given RyRs cluster, which limits Ca\textsuperscript{2+} sparks and waves, favoring smaller, nonspark Ca\textsuperscript{2+} release events. During an action potential and rapid Na\textsuperscript{+} influx, local NCX may boost [Ca\textsuperscript{2+}]\textsubscript{junct} and the efficacy of local SR Ca\textsuperscript{2+} release, especially in the latent period before LTCC opening. [Ca\textsuperscript{2+}]\textsubscript{junct} could exceed that in bulk cytosol ([Ca\textsuperscript{2+}]\textsubscript{bulk}) even during diastole. This is because spontaneous SR Ca\textsuperscript{2+} release (leak) and Ca\textsuperscript{2+} influx caused by stochastic openings of LTCCs may create a standing [Ca\textsuperscript{2+}] gradient between the cleft (where Ca\textsuperscript{2+} influx and release happen) and the bulk cytosol (where most of SR Ca\textsuperscript{2+} uptake occurs). Indeed, there is functional evidence for elevated [Ca\textsuperscript{2+}]\textsubscript{junct} in NCX knockout cardiac myocytes, which causes reduced LTCC availability and higher ability of the remaining LTCC to trigger SR Ca\textsuperscript{2+} release.

Despite its importance, methods to directly measure [Ca\textsuperscript{2+}]\textsubscript{junct} are only beginning to be developed. Indirect estimates based on the dynamic properties of Ca\textsuperscript{2+}-dependent inactivation of LTCC and NCX current during electrically triggered SR Ca\textsuperscript{2+} release suggest that [Ca\textsuperscript{2+}]\textsubscript{junct} peaks within 10 to 20 ms and reaches tens or even hundreds of micromolar. In contrast, the global Ca\textsuperscript{2+} transient reaches a maximum of \(\approx 1 \mu\text{mol/L} \) within \(\approx 70 \text{ to } 80 \text{ ms}\). Although strongly supporting the theory of local control of ECC, such measurements are indirect, difficult to calibrate, depend on the specific membrane localization of these transporters, and provide no information on diastolic [Ca\textsuperscript{2+}]\textsubscript{junct}. Our aim was to construct novel Ca\textsuperscript{2+} sensors that are specifically targeted to the sarcolemma–SR junctions and thus report local [Ca\textsuperscript{2+}]\textsubscript{junct}. We used these sensors to assess the dynamic changes in [Ca\textsuperscript{2+}]\textsubscript{junct} during ECC and to demonstrate the existence of a standing [Ca\textsuperscript{2+}] gradient during diastole between the cleft and the bulk cytosol.

Methods

Detailed Methods are included in the Online Data Supplement.

Mutagenesis of GCaMP2-Based Ca\textsuperscript{2+} Sensors, Cleft-Targeting, Plasmid Construction, and Expression in Adenoviral Vectors

The genetically encoded Ca\textsuperscript{2+} sensors GCaMP2.2 and GCaMP2.2Low were constructed by introducing the T203V and, respectively both T203V and D133E mutations in GCaMP2. For targeting to the junctional cleft, GCaMP2.2 and GCaMP2.2Low were fused to the N terminus of FKBP12.6 through a peptide linker. The GCaMP2.2 constructs were subcloned into the pshuttleCMV vector using Bgl II and Not I restriction sites. Subsequent adenovirus generations and amplifications were done using the Adeasy system (Agilent).

Protein Expression and Purification

Tagged and untagged GCaMP2.2Low sensors were cloned into a pRSET expression vector and amplified in BL21 Star (DE3) pLysS cells, and then purified using Profinity IMAC Ni Charged Resins (Bio-Rad) and further subjected to size exclusion chromatography.

Ventricular Myocyte Isolation, Culture, and Adenoviral Infection

All animal protocols were approved by the animal welfare committee at University of California, Davis, and conform to the National Institutes of Health Publication No 85-23 (revised in 1996). Rat ventricular myocytes were isolated by digestion with collagenase, cultured in supplemented M199 media, and infected with adenoviruses expressing one of the GCaMP2.2 sensors. For some experiments, freshly isolated myocytes were permeabilized with 50 µg/mL saponin for 3 minutes. All experiments were done at room temperature.

Fluorescence Measurements

GCaMP2.2s fluorescence was recorded with a Live-5 laser scanning confocal microscope (excitation, 488 nm; emission, >505 nm). Ca\textsuperscript{2+} transients were measured in linescan mode (2 ms/line). In other experiments, 2-dimensional images were taken 1 s apart.

Statistical Analysis

The statistical differences between groups were determined using the Student t test.

Results

Low-Affinity GCaMP-Based Genetically Encoded Ca\textsuperscript{2+} Indicator

GCaMPs are genetically encoded Ca\textsuperscript{2+} indicators consisting of a circularly permutated, enhanced green fluorescent protein that is flanked by calmodulin at the C terminus and by the calmodulin-binding peptide myosin light-chain kinase M13 at the N terminus (Figure 1A) and have been used to detect [Ca\textsuperscript{2+}] in vivo. The T203V mutation increases the brightness and dynamic range of GCaMP2 (GCaMP2.2), and we used this as a starting point to screen mutations that decrease the Ca\textsuperscript{2+}-binding affinity of GCaMP2.2, while maintaining brightness and dynamic range. EF-hand loop mutational screening identified GCaMP2.2Low, which contains a D133E mutation in the EF-hand loop IV of calmodulin (Figure 1A). GCaMP2.2Low has a similar baseline fluorescence and dynamic range as GCaMP2.2, but its affinity for Ca\textsuperscript{2+} binding is 10-fold lower than that of GCaMP2.2 (K\textsubscript{d} = 5 µmol/L versus 450 nmol/L; Figure 1B).

GCaMP2.2-FKBP12.6 Ca\textsuperscript{2+} Sensors Report [Ca\textsuperscript{2+}]\textsubscript{junct} in the Space Between LTCC and RyR

Our strategy for targeting Ca\textsuperscript{2+} sensors to a specific subcellular microdomain (the cleft) is to attach GCaMP2.2 and our new GCaMP2.2Low to a molecule that specifically targets to that microdomain. On the basis of their Ca\textsuperscript{2+} affinities (Figure 1B), we expected that GCaMP2.2 would be useful for measuring diastolic [Ca\textsuperscript{2+}], whereas GCaMP2.2Low could record the large Ca\textsuperscript{2+} transients expected in the
To target GCaMP2.2s to the junctions, we fused them to the N terminus of FKBP12.6 (Figure 1A). FKBP12.6 is an endogenous protein that binds with high affinity (K_d ≈ 1 nmol/L), specificity, and 1:1 stoichiometry to RyR2 monomers but does not greatly influence RyR2 function.31 Our previous work showed that fluorescent-tagged sensors are positioned to measure local cleft [Ca^{2+}]_{cleft} and, respectively, bulk cytosolic [Ca^{2+}]_{bulk} and are highly sensitive to Ca^{2+} in intact myocytes. A, Example of myocytes infected with adenoviruses expressing untagged GCaMP2.2Low (left) and GCaMP2.2Low-FKBP12.6 (right). Bottom, The fluorescence profile along the longitudinal axis of the myocyte for the area shown in the white square in the top Right and left. Limited areas from each myocyte; entire myocyte images are in Online Figure V. B, Ca^{2+}-sensitivity of GCaMP2.2Low-FKBP12.6 in intact cells. A resting myocyte expressing GCaMP2.2Low-FKBP12.6 was imaged first under control conditions (Ba). The cell was then exposed to Ca^{2+}-free external solution in the presence of 10 µmol/L ionomycin (Bb). This resulted in a significant fluorescence drop (F_{min}). Then, the cell was Ca^{2+} overloaded in a 0Na+/1 mmol/L Ca^{2+} external solution, which favors Ca^{2+} entry via both ionomycin and reverse mode Na^{+}/Ca^{2+} exchange, to saturate the sensor and determine F_{max} (Bc). The F_{max}/F_{min} ratio for this cell is 6.2. [Ca^{2+}]_{bulk} indicates [Ca^{2+}] in the bulk cytosol; and [Ca^{2+}]_{cleft}, local [Ca^{2+}] in the junctional cleft.
FKBP12.6 binds in a striated pattern along T-tubules at the sites of Ca\(^{2+}\) spark initiation, and that binding is prevented by preincubation with nonfluorescent FKBP12.6.\(^{31}\) Linking FKBP12.6 to the GCaMP sensors does not significantly affect their Ca\(^{2+}\) affinity (Figure 1B) or Ca\(^{2+}\)-binding kinetics (Online Table I). Saponin-permeabilized myocytes exposed to 100 nmol/L purified untagged GCaMP2.2 show uniform cytosolic distribution of the sensor (Figure 1Ca; Online Figure I shows the entire cell). In contrast, GCaMP2.2-FKBP12.6 has a striated fluorescence pattern (Figure 1Cb) like that seen for purified FKBP12.6 tagged with a small molecule fluorophore,\(^{31}\) which suggests that the sensor is appropriately localized at the z-line where RyRs and clefts are situated. To test the specificity of GCaMP2.2-FKBP12.6 binding at RyRs further, we exposed permeabilized myocytes to GCaMP2.2-FKBP12.6 in the presence of excess nonfluorescent FKBP12.6 to saturate RyRs (Figure 1Cc). In this case, GCaMP2.2-FKBP12.6 shows uniform cytosolic fluorescence. These data suggest that the FKBP12.6-tagged GCaMP2.2 sensors bind with high selectivity to RyRs, which are mainly cleft localized.

We next assessed the affinity of GCaMP2.2-FKBP12.6 binding to RyRs by performing sensor wash-in and wash-out experiments in permeabilized myocytes (Figure 1D). The rate constants for the dissociation (k\(_{\text{off}}\)) and association (k\(_{\text{on}}\)) of the sensor from/to RyRs were calculated from exponential fits of the decline or GCaMP2.2-FKBP12.6 fluorescence on removing the sensor from the solution (k\(_{\text{decline}}\) = k\(_{\text{off}}\)), and the GCaMP2.2-FKBP12.6 fluorescence decline on addition of the sensor to RyRs was calculated from k\(_{\text{on}}\). The dissociation constant of the RyR-GCaMP2.2-FKBP12.6 complex (K\(_{\text{D}}\)) was calculated as k\(_{\text{off}}\)/k\(_{\text{on}}\) and yielded a value of 15.5±3.3 nmol/L. A similar calculation showed that GCaMP2.2Low-FKBP12.6 binds to RyRs with a K\(_{\text{D}}\) of 43.8±3.3 nmol/L. Although these affinities are lower than previously reported for FKBP12.6 alone,\(^{31}\) GCaMP2.2-FKBP12.6 and GCaMP2.2Low-FKBP12.6 bind RyR with relatively high affinity.

We then calibrated the [Ca\(^{2+}\)] dependence of the sensors in the myocyte environment (Figure 1E). The dynamic range of the sensors in myocytes was comparable with that measured in vitro (≈6-fold), but the apparent Ca\(^{2+}\) affinity was slightly lower in myocytes for both GCaMP2.2-FKBP12.6 and GCaMP2.2 than in vitro (K\(_{\text{D}}\)=1.2 and 1.3 mmol/L, respectively). GCaMP2.2Low and GCaMP2.2Low-FKBP12.6 also exhibited roughly a 2-fold decrease in apparent affinity in the myocyte environment (K\(_{\text{D}}\)=11 mmol/L). Although GCaMP2.2-FKBP12.6 could increase Ca\(^{2+}\) buffering in the cleft, such effect is expected to be small and have limited effect for 2 reasons. First, there would be at most 1 GCaMP per RyR, and this is minor when compared with the intense >100:1 buffering in the cleft environment.\(^{3}\) Endogenous Ca\(^{2+}\)-binding sites include calmodulin and divalent binding sites on RyR, L-type Ca\(^{2+}\) channels and other cleft proteins, membrane sites, and ATP. Second, buffering in the cleft will not alter the steady state [Ca\(^{2+}\)]\(_{\text{Cleft}}\) at rest and could only slow achievement of steady state by 1 to 5 ms during channel opening or closing.\(^{5}\)

After characterizing our junctional cleft-targeted Ca\(^{2+}\) sensors, we expressed them in intact rat cardiac myocytes by adenoviral infection. Myocytes were used for experiments 20 to 24 hours later. Membrane staining with Di-8-ANNEPS indicated that myocytes retain the T-tubules at which most of the junctions reside, under the culture conditions (Online Figure III). Moreover, a stringent test for normal junctional function used transverse line scan images using Fluo-4 (where spatial drop-out or delayed central activation could indicate altered junctional coupling). The results demonstrated that normal transverse synchrony of SR Ca\(^{2+}\) release was well preserved in myocytes expressing the GCaMP sensors after 24 hours culture (Online Figure IV). There was no decrement in either the uniformity of local transverse release activation or rate of rise of the Ca\(^{2+}\) transients. Thus, the clefts where the GCaMP2.2-FKBP12.6 sensors are targeted function normally during ECC.

FKBP12.6-tagged sensors express in a striated pattern, with intensity maxima spaced ≈2 µm apart (Figure 2A; full images are shown in Online Figure V). This strongly supports the conclusion that the FKBP12.6-tagged sensors are targeted to RyR at the z-line, as in permeabilized cells. In contrast, the untagged sensors show a rather uniform cytosolic distribution (Figure 2A). To test the Ca\(^{2+}\) responsiveness of the new sensors when expressed in intact cells, we first depleted myocytes of Ca\(^{2+}\) (using ionomycin and Ca\(^{2+}\)-free solution), to determine the minimum sensor fluorescence (F\(_{\text{min}}\); Figure 2Bb versus Figure 2Ba). Then, myocytes were Ca\(^{2+}\)-overloaded by restoring extracellular Ca\(^{2+}\) in Na\(^{-}\)-free solution to assess maximum fluorescence (F\(_{\text{max}}\)) just as hypercontracture begins (Figure 2Bc). Importantly, GCaMP2.2Low and GCaMP2.2Low-FKBP12.6 have a similar dynamic range, F\(_{\text{max}}\)/F\(_{\text{min}}\) in myocytes (5.5±0.6; n=7 for GCaMP2.2 and 5.7±0.5; n=9 for GCaMP2.2-FKBP12.6).
These numbers agree well with those in Figure 1E. Thus, our novel FKB12.6-tagged Ca²⁺ indicators are highly sensitive to Ca²⁺ and are positioned to measure local cleft [Ca²⁺]_{Cleft} versus untagged sensors reporting bulk cytosolic [Ca²⁺]_{Bulk} in intact myocytes.

**Larger and Faster Local Ca²⁺ Transients in the Junctional Space Versus Bulk Cytosol**

We expressed the low-affinity untagged and FKB12.6-tagged sensors to measure Ca²⁺ transients in the bulk cytosol and junctional cleft, respectively (Figure 3). Myocytes were field-stimulated at 0.5 Hz, and Ca²⁺ transients were measured in linescan mode using a laser scanning confocal microscope. Figure 3A shows representative Ca²⁺ transients recorded by GCaMP2.2Low and GCaMP2.2Low-FKB12.6. Junctional cleft transients recorded by GCaMP2.2Low-FKB12.6 are ≈2-fold larger (Figure 3B) and have a much faster upstroke (time-to-peak, 46±5 versus 90±7 ms; Figure 3C) and decay (decay time=254±19 versus 421±42 ms; Figure 3D) when compared with global Ca²⁺ transients measured by the untargeted GCaMP2.2Low. This is encouraging (see Discussion section of this article for inferred [Ca²⁺] values), but based on theoretical models and indirect cleft Ca²⁺ transient assessments, one would expect a much larger difference in both amplitude and kinetics between the targeted and the untargeted sensor. An obvious explanation is that the kinetics of local [Ca²⁺] change in the cleft are likely to be fast, and GCaMP sensors lack the temporal resolution to detect such rapid kinetics. The dissociation rate constant for GCaMP2.2Low (∼6 s⁻¹; Online Table 1) is consistent with this notion. Although this new low-affinity Ca²⁺-sensor is not fast enough to report [Ca²⁺]_{Cleft} dynamics during ECC accurately, it may still be useful in determining relative changes in [Ca²⁺]_{Cleft} with acute treatments (eg, slower or larger). Targeted GCaMP2.2Low may also provide more accurate values of local [Ca²⁺], in microdomains with local Ca²⁺ flux rates that are lower than in stratified muscle junctions, where local Ca²⁺ flux rates may be among the highest in nature.

On β-adrenergic activation with isoproterenol (ISO; Online Figure VI), untargeted GCaMP2.2Low readily detects the expected large increase in Ca²⁺ transient amplitude and 2-fold acceleration of [Ca²⁺] decline because of enhanced SR Ca²⁺ uptake. The cleft-targeted sensor Ca²⁺ transient decays much faster than the untargeted sensor under control conditions but fails to accelerate with ISO, despite a similar doubling of amplitude. Because [Ca²⁺]_{Cleft} is expected to sense released Ca²⁺ and diffusion from the cleft to cytosol, rather than SR Ca²⁺ uptake rate (which is distributed throughout the cytosol), this is exactly what one would expect. Thus, the cleft-targeted GCaMP2.2Low is more sensitive to Ca²⁺ release at the cleft than to transport by SR Ca²⁺-ATPase (SERCA) and differs qualitatively from bulk [Ca²⁺] sensors. This relates to Ca²⁺ sparks that decay primarily via diffusion from the source, with minor influence by SERCA function, despite much of the indicator signal coming from outside the cleft. In contrast, global [Ca²⁺], decline is dictated almost entirely by Ca²⁺ transport out of the cytosol.

**Standing Diastolic [Ca²⁺] Gradient Between the Junctional Space and Bulk Cytosol**

During diastole, Ca²⁺ leak from the SR or Ca²⁺ influx across sarcolemma may raise local [Ca²⁺]_{Cleft} above [Ca²⁺]_{Bulk} (distant from Ca²⁺ sources). Moreover, the high density of both L-type Ca²⁺ channels and RyR2 at the cleft when compared with the broader distribution of the transporters that remove Ca²⁺ from the cytosol (SR Ca²⁺-ATPase and Na⁺/Ca²⁺ exchange) could allow even modest Ca²⁺ leak through these channels to produce a standing [Ca²⁺] gradient (Figure 4A, left). Increases in SR Ca²⁺ leak have been implicated in pathological states, where [Ca²⁺]_{Cleft} could be preferentially elevated. We used our targeted GCaMP2.2 to test for such diastolic standing [Ca²⁺] gradients between the cleft and the cytosol directly.

On blocking Ca²⁺ fluxes into the cleft, [Ca²⁺] should decline in both compartments, and importantly, any [Ca²⁺]_{Cleft} versus [Ca²⁺]_{Bulk} gradient should dissipate (Figure 4A, right). This maneuver is valuable so that we can define a point (F₀) where both the cytosolic and targeted GCaMP2.2 sense the same [Ca²⁺], Figure 4B and 4C shows how [Ca²⁺]_{Cleft} and [Ca²⁺]_{Bulk} decline when we abruptly block RyRs with 1 mmol/L tetrodactin and both LTCC and NCX by rapidly switching the external solution to a 0Na⁺/0 Ca²⁺ solution. Both [Ca²⁺]_{Cleft} and [Ca²⁺]_{Bulk} dropped rapidly after the application of tetrodactin and 0Na⁺/0 Ca²⁺ solution, reaching the F₀ steady state (where [Ca²⁺]_{Cleft}=[Ca²⁺]_{Bulk}) in 1 minute (Figure 4B and 4C, transition time from a to b). Note that [Ca²⁺] decline was much larger for GCaMP2.2-FKB12.6. These results suggest that the initial diastolic [Ca²⁺]_{Cleft} was higher (F/F₀=1.8) than [Ca²⁺]_{Bulk} (F/F₀=1.2). In both cases, fluorescence was restored on return to the normal bath solution (time point c in Figure 4B and 4C).

Mean data confirm that the inhibition of SR and sarcolemma Ca²⁺ fluxes in the cleft produces a significantly larger decline in the fluorescence of GCaMP2.2-FKB12.6, which reports local [Ca²⁺]_{Cleft} versus the untagged GCaMP2.2, which reports [Ca²⁺]_{Bulk} (ΔF/F₀=0.55±0.10 for GCaMP2.2-FKB12.6 versus 0.13±0.02 for GCaMP2.2; Figure 4D). This indicates that there is indeed a standing [Ca²⁺] gradient during diastole between the junctional cleft and the bulk cytosol. Assuming that diastolic [Ca²⁺]_{Bulk}=100 nmol/L (before tetracaine, 0Na⁺/0Ca²⁺) and using data in Figure 1E, we calculated that diastolic [Ca²⁺]_{Cleft} is 194±28 nmol/L.

Another way of reducing diastolic SR Ca²⁺ leak is to block SERCA with thapsigargin (Figure 5A). SERCA inhibition slowly unloads the SR, which is paralleled by a slow decrease in SR Ca²⁺ leak. As expected, with 10 μmol/L thapsigargin and 0Na⁺/0 Ca²⁺ solution, the fluorescence signal in myocytes expressing either GCaMP2.2 or GCaMP2.2-FKB12.6 declined more slowly, reaching a new steady state in ≈10 minutes (Figure 5B). A similar time course was previously demonstrated for thapsigargin-induced cessation of SR Ca²⁺ leak, measured as the rate of decline in SR [Ca²⁺]₀. Similar
Ger time in Ca2+-free solution), but the difference between thapsigargin versus tetracaine (presumably because of long lemmal Ca 2+ fluxes to Ca 2+ entry into the cleft, we did separate experiments where only sarcolemmal Ca2+ fluxes are blocked (0Na+/0 Ca2+ solution). In this case, the decrease in the sensor fluorescence was similar for the FKBP12.6-tagged and the untagged sensor (−ΔF/F0=0.22±0.03 for GCaMP2.2-FKBP12.6 versus 0.17±0.02 for GCaMP2.2; Figure 5C). In contrast, inhibition of SR Ca2+ leak alone resulted in a significantly larger drop in the GCaMP2.2-FKBP12.6 fluorescence versus the untagged sensor (−ΔF/F0=0.55±0.11 versus 0.24±0.07; Figure 5C). These results indicate that most of the Ca2+ entering the junctional cleft during diastole comes from the SR and SR Ca2+ leak is the main factor in setting a diastolic [Ca2+]i gradient between the cleft and the bulk cytosol.

Discussion

In many cell types, [Ca2+] is regulated and signals differently in various subcellular microdomains, which greatly enhances its versatility as a secondary messenger. Recognition of such subcellular regulation stimulated us to develop methods for measuring local [Ca2+] in microdomains. Here, we attached the genetically encoded Ca2+ sensor GCaMP2.2 and a newly developed lower Ca2+ affinity variant GCaMP2.2Low to the N terminus of FKBP12.6 to construct novel Ca2+ sensors that bind specifically to RyRs. Our data indicate that GCaMP2.2-FKBP12.6 and GCaMP2.2Low-FKBP12.6 have a high affinity and selectivity for RyRs although slightly reduced when compared with untagged FKBP12.6. In cardiac myocytes, RyRs are localized predominantly in the junctional SR membrane, where the SR comes into close proximity of the external sarcolemma (the dyads). Because of this specific targeting, the FBP12.6-tagged sensors report local [Ca2+] in the small dyadic cleft, [Ca2+]Cleft. This local [Ca2+]Cleft controls to RyR blockade with tetracaine, the fluorescence decline was significantly larger for GCaMP2.2-FKBP12.6 versus untagged GCaMP2.2 (∆F/F0=0.77±0.11 versus 0.41±0.06; Figure 5C). The overall decline in [Ca2+]i was larger for thapsigargin versus tetracaine (presumably because of longer time in Ca2+-free solution), but the difference between cleft and bulk was similar for both methods (∆F/F0=0.36 versus 0.42).

To assess the relative contribution of the SR and sarcolemmal Ca2+ fluxes to Ca2+ entry into the cleft, we did separate experiments where only sarcolemmal Ca2+ fluxes are blocked (0Na+/0 Ca2+ solution). In this case, the decrease in the sensor fluorescence was similar for the FKBP12.6-tagged and the untagged sensor (∆F/F0=0.22±0.03 for GCaMP2.2-FKBP12.6 versus 0.17±0.02 for GCaMP2.2; Figure 5C). In contrast, inhibition of SR Ca2+ leak alone resulted in a significantly larger drop in the GCaMP2.2-FKBP12.6 fluorescence versus the untagged sensor (∆F/F0=0.55±0.11 versus 0.24±0.07; Figure 5C). These results indicate that most of the Ca2+ entering the junctional cleft during diastole comes from the SR and SR Ca2+ leak is the main factor in setting a diastolic [Ca2+] gradient between the cleft and the bulk cytosol.
and is influenced by both RyRs and L-type Ca\(^{2+}\) channels. Therefore, [Ca\(^{2+}\)\(_{\text{Cleft}}\)] critically affects myocyte function at rest (during diastole) and during electric excitation. Despite its importance, methods to measure [Ca\(^{2+}\)\(_{\text{Cleft}}\)] directly are only now becoming available.\(^{21,22}\) This key parameter has typically been inferred from the dynamics of NCX\(^{12,24}\) and L-type Ca\(^{2+}\) currents\(^{22,23}\) in electrically stimulated myocytes, but no such estimates could be made for diastolic [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\). Our novel Ca\(^{2+}\) sensors allow direct measurement of [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\) both during ECC (the low-affinity sensor GCaMP2.2Low-FKBP12.6) and at rest (GCaMP2.2-FKBP12.6). However, the extremely rapid changes of [Ca\(^{2+}\)\(_{\text{Cleft}}\)] expected during ECC are too fast to be captured accurately by even the 10 \(\mu\)mol/L \(K_d\) sensor although they are likely to be useful to assess relative changes in [Ca\(^{2+}\)\(_{\text{Cleft}}\)] during perturbations that might alter the amplitude or kinetics of [Ca\(^{2+}\)]\(_{\text{bulk}}\) changes during ECC (as we have seen for ISO stimulation).

We use FKBP12.6 to target the sensors because of the high affinity and selectivity for RyR2 in cardiac myocytes.\(^{31}\) A limitation with this approach is that FKBP12.6 can also influence RyR channel gating, stabilizing the closed state.\(^{35}\) FKBP12.6 overexpression in myocytes can reduce spontaneous Ca\(^{2+}\) spark frequency and either increase or decrease Ca\(^{2+}\) transient amplitude.\(^{36,37}\) Thus, using FKBP12.6 to target these Ca\(^{2+}\) sensors may reduce diastolic RyR2 opening. However, in studies measuring simultaneously FKBP12.6 binding to RyR2 and Ca\(^{2+}\) sparks, saturating RyR2 with FKBP12.6 only reduced Ca\(^{2+}\) spark frequency by 18%.\(^{38}\) This effect would mean that we are probably slightly underestimating diastolic [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\).

A fraction (\(\simeq 15%-20\%\)) of RyRs are located outside the sarcolemma–SR junctions in rat myocytes.\(^{39}\) Thus, the GCaMP2.2-FKBP12.6 signal is not generated entirely by [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\), but is slightly contaminated by [Ca\(^{2+}\)]\(_{\text{bulk}}\) at such nonjunctional RyRs. Of note, local [Ca\(^{2+}\)] at nonjuncti- tional RyRs may also be relevant, even if less influential on sarcolemmal currents. The adenoviral expression of GCaMP sensors in cardiac myocytes could alter regulation of dyad structure or function. Although cleft dimensions were not directly measured (eg, by electron microscopy), the FKBP12.6-targeted sensors are highly localized along T-tubules (of which \(50\%\) are in dyads in rat myocytes)\(^{9}\) and normal synchronous transverse activation during ECC was maintained (Online Figure IV). Therefore, we assume that the ultrastructure of the junctional cleft was not appreciably altered by the experimental conditions. FKBP12.6-GCaMP2.2 also has to be studied in a practical range of protein expression. If one waits too long or transfects too aggressively, one can express enough FKBP12.6-targeted protein to more than saturate the \(\simeq 1\) \(\mu\)mol/L of RyR-binding sites. In that case, the excess sensor will not be effectively targeted. Any amount of untargeted GCaMP2.2-FKBP12.6 (as above) would cause underestimation of the difference between [Ca\(^{2+}\)\(_{\text{Cleft}}\)] and [Ca\(^{2+}\)\(_{\text{bulk}}\)]. This means that our [Ca\(^{2+}\)\(_{\text{Cleft}}\)] measurements and the diastolic [Ca\(^{2+}\)] gradient are lower limit estimates. True [Ca\(^{2+}\)\(_{\text{Cleft}}\)] would be larger. On balance, with awareness of these challenges with respect to interpretation, we think that the advantages of targeting sensors to the cleft this way outweigh the limitations and do not affect any of our conclusions. Potential enhancements in live cell imaging resolution that selectively visualize such local domains would complement approaches like ours with locally targeted sensors.\(^{22}\)

**[Ca\(^{2+}\)]\(_{\text{Cleft}}\) and [Ca\(^{2+}\)]\(_{\text{bulk}}\) in Cardiac Myocytes**

Our novel cleft-targeted sensor GCaMP2.2Low-FKBP12.6 reported a larger and faster rise in [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\) versus [Ca\(^{2+}\)\(_{\text{bulk}}\)] during electric excitation of myocytes, in good agreement with previous estimates from indirect measurements.\(^{9-12}\) Weber et al\(^{24}\) inferred the submembrane [Ca\(^{2+}\)] near NCX during a normal action potential as peaking at \(>3.2\) \(\mu\)mol/L within \(\leq 32\) ms (versus a global Ca\(^{2+}\) transient that peaks at 1.1 \(\mu\)mol/L in 81 ms). However, [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\) ought to be much higher than submembrane [Ca\(^{2+}\)]. Acsai et al\(^{12}\) estimated the Ca\(^{2+}\) transient near Ca\(^{2+}\) release sites from the kinetics of L-type Ca\(^{2+}\) current and from a fraction of NCX current. They calculated that this local Ca\(^{2+}\) transient has 10 to 15 \(\mu\)mol/L in amplitude, peaks at \(\approx 10\) ms after depolarization and recovers within \(\approx 50\) ms. In contrast, the global Ca\(^{2+}\) transient reached a maximum of \(1\) \(\mu\)mol/L within \(\approx 70\) to 100 ms. Estimates of [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\) based on geometry, diffusion, and Ca\(^{2+}\) flux measurements suggest even higher (100 \(\mu\)mol/L) and faster [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\) changes during electric excitation.\(^{11}\) On the basis of measured diastolic [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\) and [Ca\(^{2+}\)]\(_{\text{bulk}}\) (194 and 100 \(\mu\)mol/L, respectively; Figure 4E), the characteristics of GCaMP2.2Low and GCaMP2.2Low-FKBP response to Ca\(^{2+}\) in myocytes (Figure 1E), and the Ca\(^{2+}\) transient amplitude (Figure 3C), we calculated that peak [Ca\(^{2+}\)]\(_{\text{bulk}}\) is 560\(\pm\)73 \(\mu\)mol/L, whereas peak [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\) is \(\approx 2\)-fold larger (1272\(\pm\)199 \(\mu\)mol/L). This peak [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\) is much smaller than previous estimates. The time-to-peak of [Ca\(^{2+}\)\(_{\text{Cleft}}\)\(_{\text{bulk}}\)] was 46\(\pm\)5 ms (versus 90\(\pm\)7 ms for [Ca\(^{2+}\)]\(_{\text{bulk}}\)), slightly longer than the estimates based on NCX and L-type Ca\(^{2+}\) current. We think that these differences in the amplitude and dynamics of cleft Ca\(^{2+}\) transients versus previous estimates are mostly because of the relatively slow kinetics of Ca\(^{2+}\) binding and unbinding of the GCaMP2.2 sensors (Online Table I). Thus, although GCaMP2.2Low-FKBP12.6 detects larger and faster cleft Ca\(^{2+}\) transients versus the global sensor and can detect changes in amplitude and kinetics (eg, with ISO), much faster indicators will be required for accurate [Ca\(^{2+}\)\(_{\text{Cleft}}\)]\(_{\text{bulk}}\) measurement during the dynamics of active SR Ca\(^{2+}\) release in heart.

Using GCaMP2.2-FKBP12.6, we could directly assess diastolic [Ca\(^{2+}\)\(_{\text{Cleft}}\)] a parameter not previously measured. We demonstrated that during diastole, [Ca\(^{2+}\)\(_{\text{Cleft}}\)] is \(>90\) \(\mu\)mol/L higher than [Ca\(^{2+}\)]\(_{\text{bulk}}\). This standing diastolic [Ca\(^{2+}\)]\(_{\text{Cleft}}\) gradient indicates that Ca\(^{2+}\) extrusion from the cleft (via functionally located NCX and diffusion into bulk cytosol) cannot keep up with Ca\(^{2+}\) entry into the cleft. Our data show that diastolic cleft Ca\(^{2+}\) influx is caused mainly by the SR Ca\(^{2+}\) leak (versus entry through LTCC). At the same time, elevated diastolic [Ca\(^{2+}\)]\(_{\text{Cleft}}\) can reduce LTCC availability (via Ca\(^{2+}\)-dependent inactivation) and alter RyR gating to increase their activity by direct RyR activation and also indirectly via activation of CaMKII followed by CaMKII-dependent RyR phosphorylation.\(^{36}\) Increased RyR opening leads to a larger SR Ca\(^{2+}\) leak...
and a higher risk for generation of propagating Ca\(^{2+}\) waves, which are a known risk for arrhythmias. The balance of Ca\(^{2+}\) fluxes in the cleft is altered under pathological conditions (eg, in heart failure SR Ca\(^{2+}\) leak and Na\(^+/\)Ca\(^{2+}\) exchange function are increased).\(^4\)

The high diastolic [Ca\(^{2+}\)]\(_{\text{Cleft}}\) may also have important effects on local signaling cascades beyond the sphere of ECC. Indeed, there is much indirect evidence that local [Ca\(^{2+}\)]\(_i\) affects on local signaling cascades beyond the sphere of ECC. We have also provided measures of a normal standing [Ca\(^{2+}\)]\(_i\) gradient during diastole between the environments which are a known risk for arrhythmias. The balance of Ca\(^{2+}\) leak is known to be enhanced, the higher local level of diastolic [Ca\(^{2+}\)]\(_{\text{Cleft}}\) may have an important effect on these (and other) broader signaling pathways.

In summary, we have developed sensors for measuring [Ca\(^{2+}\)]\(_{\text{Cleft}}\) versus [Ca\(^{2+}\)]\(_{\text{bulk}}\) and demonstrated dynamic differences during ECC. We have also provided measures of a normal standing [Ca\(^{2+}\)]\(_i\) gradient during diastole between the functional cleft and the bulk cytosol and showed that this gradient is set mainly by the SR Ca\(^{2+}\) leak.

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Disclosures
None.

References


### Novelty and Significance

**What Is Known?**

• Ca2+ is regulated and signals differently in various subcellular microdomains, which greatly enhances its second messenger versatility.

• In the heart, sarcoplasmic reticulum Ca2+ release is controlled by local [Ca2+]i in the junctional cleft, the small space between sarcolemma and junctional sarcoplasmic reticulum.

• This local cleft [Ca2+]i is thought to be important in not only the regulation of excitation–contraction coupling but also to downstream Ca2+-dependent signaling (eg, via calmodulin, calcineurin and CaMKII).

**What New Information Does This Article Contribute?**

• We constructed novel Ca2+-sensors that are targeted to microdomains rich in ryanodine receptors.

• In cardiac myocytes, the novel Ca2+-sensors are positioned to measure local Ca2+ in the junctional cleft.

• There is a standing Ca2+ gradient during diastole between the junctional cleft and bulk cytosol in cardiac myocytes, which could influence local Ca2+-dependent signaling within the cleft.

Ca2+ is a universal second messenger involved in activation/regulation of diverse cellular processes. To allow such versatility, Ca2+ is regulated and signals differently in various subcellular microdomains, which generated a large interest in developing methods for measuring local rather than bulk Ca2+. Here, we targeted novel Ca2+-sensors to microdomains rich in ryanodine receptors. This was accomplished by fusing the genetically encoded Ca2+-sensor GCaMP2.2 and a lower Ca2+-affinity variant GCaMP2.2Low to FKBP12.6, a protein that binds with high affinity and selectivity to ryanodine receptors. In cardiac myocytes, these sensors report local [Ca2+] in the small junctional cleft between the plasmalemma and junctional sarcoplasmic reticulum ([Ca2+]Cleft), a parameter that critically affects heart function and dysfunction. Using these sensors, we demonstrate that [Ca2+]Cleft reaches higher levels with faster kinetics than global [Ca2+]i, during excitation–contraction coupling. We also show that there is a substantial standing diastolic [Ca2+]Cleft gradient between [Ca2+]Cleft and bulk [Ca2+], mainly because of ryanodine receptor–dependent Ca2+ leak. Such [Ca2+]Cleft characteristics may have important effect on local Ca2+-dependent signaling.
Junctional Cleft $[\text{Ca}^{2+}]_i$ Measurements Using Novel Cleft-Targeted $\text{Ca}^{2+}$ Sensors
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Supplemental Material

Detailed Methods

Generation of junctional cleft-targeted Ca^{2+} sensors and plasmid construction. The genetically encoded Ca^{2+} sensor GCaMP2.2 was fused to the N-terminus of FKBP12.6 through the peptide linker (AP linker) GSSTSGAPAPAPAPAPAPSEF. The GCaMP2.2 coding sequence was PCR-amplified with forward primer Nhel-RSET tatggctagcatctagttgacag, and reverse primer EcoRI-LinkerAP-CaM atacgaattcagagctgagcagcagagcagagcagcagagcaga, followed by insertion at the C termini of AP linker between 0.5 µM cytochalasin D, which was shown to help preserve the T-tubular structure during short-term culture. 

Myocytes were plated on laminin-coated coverslips and cultured in supplemented M199 media containing digested with collagenase (1 mg/ml).

Protein Expression and Purification. The GCaMP2.2(Low) and GCaMP2.2(Low)-FKBP12.6 sensors were cloned into a pRSET expression vector. Overnight seed cultures of transformed BL21 Star (DE3) pLysS cells were used to inoculate (1:40 dilution) 500 mL Terrific Broth containing 100 mg/mL ampicillin. The cultures were incubated at 37ºC (250 rpm) until OD_{600} ~1.0. Then, 0.5 mM IPTG was added and cultures were incubated for 16 h at 20ºC (250 rpm). Cells were collected by centrifugation at 6000g for 10 minutes at 4ºC and resuspended in 20 mM HEPES (pH7.4) with 350 mM NaCl. Cultures were sonicated and cell debris removed by centrifugation at 20,000g for 30 minutes at 4ºC. The cleared lysate was mixed with 3 ml of Profinity IMAC Ni Charged Resins (Bio-Rad) and loaded onto a 0.8×4cm chromatography column (Bio-Rad), washed 3 times with 20 mM HEPES (pH 7.4) containing 350 mM NaCl, with each wash followed by low speed centrifugation at 800×g for 30 seconds. Proteins were eluted with 20 mM HEPES (pH 7.8) containing 350 mM NaCl and 300 mM imidazole buffer. Proteins were further subjected to size exclusion chromatography on a Superdex200 column (16/60; GE Healthcare) and loaded onto a 0.8×4cm chromatography column (Bio-Rad), washed 3 times with 20 mM HEPES (pH 7.4) with 350 mM NaCl. Cultures were sonicated and cell debris removed by centrifugation at 20,000g for 30 minutes at 4ºC. The cleared lysate was mixed with 3 ml of Profinity IMAC Ni Charged Resins (Bio-Rad) and loaded onto a 0.8×4cm chromatography column (Bio-Rad), washed 3 times with 20 mM HEPES (pH 7.4) containing 350 mM NaCl, with each wash followed by low speed centrifugation at 800×g for 30 seconds. Proteins were eluted with 20 mM HEPES (pH 7.8) containing 350 mM NaCl and 300 mM imidazole buffer. Proteins were further subjected to size exclusion chromatography on a Superdex200 column (16/60; GE Healthcare) equilibrated in 20 mM HEPES (pH 7.4) with 100 mM NaCl. Protein purity was checked by SDS-PAGE and the concentration was measured (Pierce BCA Protein Assay).

Expression of the sensor in an adenoviral vector. The GCaMP2.2 constructs were subcloned into the pshuttleCMV vector using Bgl II and Not I restriction sites. Subsequent adenovirus generations and amplifications were done according to the manufacturer’s instruction (Adeasy system, Agilent).

Kinetics of Ca^{2+}-GCaMP binding/unbinding. The kinetics of Ca^{2+} binding/unbinding by the newly developed sensors were measured with a KinTek stop-flow fluorometer at 20 ºC. The fluorescence was detected by a 505 long-pass filter with excitation at 488 nm. The [Ca^{2+}]_{free} was calculated using WEBMAXC EXTENDED program. For Ca^{2+} binding measurements, purified GCaMP proteins (1 µM) in a Ca^{2+}-free buffer containing 1mM EGTA, 50 mM Mops, and 100 mM KCl (pH 7.4) were mixed with various [Ca^{2+}] in the same buffer in the stop-flow fluorometer. The final [Ca^{2+}]_{free} was 1 µM for GCaMP2.2 and GCaMP2.2-FKBP12.6, and 10 µM for GCaMP2.2Low and GCaMP2.2Low-FKBP12.6. To measure the kinetics of Ca^{2+} dissociation from GCaMP2.2 and GCaMP2.2-FKBP12.6, 1 µM purified protein in a solution containing 1 µM [Ca^{2+}]_{free}, 50 mM Mops, 100 mM KCl (pH 7.4) was mixed with 10 mM EGTA in the same buffer. Similarly, for GCaMP2.2Low and GCaMP2.2Low-FKBP12.6, 1 µM purified protein in 10 µM [Ca^{2+}]_{free} were mixed with 10 mM EGTA. The stop-flow traces were fit with a mono-exponential equation to calculate the observed rate constant k_{obs}.

Ventricular myocyte isolation, culture and adenoviral infection. All animal protocols were approved by the animal welfare committee at the University of California Davis and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Isolation of rat ventricular myocytes was as previously described. Briefly, rats were anesthetized by I.P. injection of nembutal (~1 mg/g) and hearts were excised quickly, placed on a Langendorff perfusion apparatus and digested with collagenase (1 mg/ml).

Myocytes were plated on laminin-coated coverslips and cultured in supplemented M199 media containing 0.5 µM cytochalasin D, which was shown to help preserve the T-tubular structure during short-term culture. One hour after plating, the cells were infected with adenoviruses expressing one of the GCaMP2.2 sensors (MOI=100). Myocytes were used for experiments after 24 hours in culture. Membrane staining with Di-8-ANNEPS indicated that myocytes retain the T-tubules under these culture conditions (Online Fig. S1). All experiments were done at room temperature (23-25ºC). The standard Tyrode’s solution contained (in mM): 140 NaCl, 4 KCl, 1 MgCl_{2}, 10
glucose, 5 HEPES and 1 CaCl$_2$ (pH=7.4). In some experiments, myocytes were exposed to 0Na$^+$/0Ca$^{2+}$ solution. In this solution, CaCl$_2$ was omitted, 5 mM EGTA was added and NaCl was replaced equimolarly with LiCl. Note that the intracellular pH did not change significantly upon switching from normal Tyrode’s solution to 0Na$^+$/0Ca$^{2+}$ solution (Online Fig. VII).

**Myocyte permeabilization.** Freshly isolated myocytes were permeabilized with 50 µg/mL saponin for 3 minutes in internal solution containing (in mM) 115 K-aspartate, 0.56 MgCl$_2$ (free [Mg$^{2+}$]=1 mM), 10 EGTA, 5 Mg-ATP, 10 reduced glutathione, 5 Na$_2$-phosphocreatine, 5 U/mL creatine phosphokinase, 20 mM BDM and 10 HEPES (pH=7.2). Free [Ca$^{2+}$] was adjusted to various levels by adding CaCl$_2$.

**Fluorescence measurements.** Fluorescence measurements were done with a Live-5 laser scanning confocal microscope (Zeiss). GCaMP2.2 was excited with the 488 nm line of an argon laser and fluorescence was collected at >505 nm. Ca$^{2+}$ transients were measured in linescan mode (line duration =2 ms). In other experiments, 2D images were taken 1 sec apart.

**Statistical analysis.** The statistical differences between groups were determined using the student’s t-test. The data are presented as mean ± standard error. Differences were considered statistically significant when P<0.05.

**Supplemental Figures and Figure Legends**

**Online Fig. I.** Fluorescence pattern of GCaMP2.2 and GCaMP2.2-FKB12.6 in permeabilized myocytes. Rat ventricular myocytes were permeabilized with saponin (50 µg/mL for 3 min) and incubated for 10 min with 100 nM purified GCaMP2.2 (top) or GCaMP2.2-FKB12.6 (middle). In the bottom panel, permeabilized myocytes were first incubated with 10 µM non-fluorescent FKB12.6 (NF-FKB12.6) for 5 min, followed by exposure to 100 nM GCaMP2.2-FKB12.6 in the continued presence of NF-FKB12.6. Small areas from these cells are shown in Fig. 1C.

**Online Fig. II.** Rate constants of GCaMP2.2(Low)-FKB12.6 binding ($k_{on}$) to/unbinding ($k_{off}$) from RyRs in saponin-permeabilized rat myocytes. $k_{off}$ was calculated from an exponential fit of the decline in sensor fluorescence after removing the sensor from the external solution. To assess $k_{on}$, we first determined the rate constant for the increase in the myocyte fluorescence upon adding 100 nM GCaMP2.2(Low)-FKB12.6. $k_{on}$ was then calculated as $(k_{on}-k_{off})/[$GCaMP2.2(Low)-FKB12.6]. F-FKBP represents FKB12.6 labeled with Alexa Fluor 488 C5 maleimide (Ref. 29 in main text).
Online Fig. III. Myocytes retain the T-tubule network after 24 h in culture. Myocytes were cultured for 24 h as described in the Methods section, then stained with the membrane marker Di-8-ANEPPS (5 µM for 15 min) and imaged with a confocal microscope.

Online Fig. IV. The synchrony of SR Ca²⁺ release is preserved following 20-24 hours of culture and viral infection. Freshly isolated myocytes and cells cultured (20-24 hours) and infected with GCaMP-FKBP12.6 were loaded with Fluo-4 and Ca²⁺ transients were recorded with a confocal microscope in linescan mode. Of note, the Fluo-4 signal was significantly higher than that produced by the GCaMP sensors. (A) Representative Ca²⁺ transients in a freshly isolated and a cultured myocyte. The scanning line is transverse (perpendicular to the long axis of the cell). Both cells show synchronous SR Ca²⁺ release, with no areas of delayed release. (B) The maximum rate of [Ca²⁺] rise, expressed as d(F/F₀)/dt. Ten sequential Ca²⁺ transients were averaged for each myocyte. n=6 cells were recorded and analyzed for both fresh and cultured groups. Similar results were obtained from longitudinal linescans (scanning line parallel with the long axis of the myocyte).

Online Fig. V. Adenovirally-expressed GCaMP2.2(Low)-FKBP12.6 and untagged GCaMP2.2(Low) are positioned to measure local cleft [Ca²⁺]cleft and, respectively, bulk cytosolic [Ca²⁺]bulk. Representative example of myocytes infected with adenoviruses expressing untagged GCaMP2.2Low (Top) and GCaMP2.2Low-FKBP12.6 (Bottom). Small areas from these cells are shown in Fig. 2A.
Supplemental Table

**Online Table I.** Rate constants for Ca\(^{2+}\) binding and unbinding of the FKBP12.6-tagged and untagged GCaMP sensors. Data are presented as mean ± standard deviation.

<table>
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<th>Sensor</th>
<th>(k_{\text{assoc}}) (s(^{-1}))</th>
<th>+(\Delta[\text{Ca}^{2+}]) (µM)</th>
<th>(k_{\text{dissoc}}) (s(^{-1}))</th>
<th>-(\Delta[\text{Ca}^{2+}]) (µM)</th>
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<td>GCaMP2.2</td>
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<td>5.69±0.30</td>
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<tr>
<td>GCaMP2.2Low-FKBP12.6</td>
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<td>5.71±0.05</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

Supplementary Reference: