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Total internal reflectance fluorescence imaging of genetically engineered ryanodine receptor-targeted Ca^{2+} probes in rat ventricular myocytes

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ABSTRACT

The details of cardiac Ca^{2+} signaling within the dyadic junction remain unclear because of limitations in rapid spatial imaging techniques, and availability of Ca^{2+} probes localized to dyadic junctions. To critically monitor ryanodine receptors' (RyR2) Ca^{2+} nano-domains, we combined the use of genetically engineered RyR2-targeted pericam probes, (FKBP-YCaMP, K_d = 150 nM, or FKBP-GCaMP6, K_d = 240 nM) with rapid total internal reflectance fluorescence (TIRF) microscopy (resolution, ~80 nm). The punctate z-line patterns of FKBP,[2]-targeted probes overlapped those of RyR2 antibodies and sharply contrasted to the images of probes targeted to sarcoplasmic reticulum (SERCA2a/PLB), or cytosolic Fluo-4 images. FKBP-YCaMP signals were too small (~20%) and too slow (2–3 s) to detect Ca^{2+} sparks, but the probe was effective in marking where Fluo-4 Ca^{2+} sparks developed. FKBP-GCaMP6, on the other hand, produced rapidly decaying Ca^{2+} signals that: a) had faster kinetics and activated synchronous with I_{Ca}[3] but were of variable size at different z-lines and b) were accompanied by spatially confined spontaneous Ca^{2+} sparks, originating from a subset of *eager* sites. The frequency of spontaneously occurring sparks was lower in FKBP-GCaMP6 infected myocytes as compared to Fluo-4 dialyzed myocytes, but isoproterenol enhanced their frequency more effectively than in Fluo-4 dialyzed cells. Nevertheless, isoproterenol failed to dissociate FKBP-GCaMP6 from the z-lines. The data suggests that FKBP-GCaMP6 binds predominantly to junctional RyR2s and has sufficient on-rate efficiency as to monitor the released Ca^{2+} in individual dyadic clefts, and supports the idea that β-adrenergic agonists may modulate the stabilizing effects of native FKBP on RyR2.

1. Introduction

During diastolic intervals, mammalian cardiomyocytes generate focal Ca^{2+} releases, "Ca^{2+} sparks" [1], that are coordinated by I_{Ca} to develop sarcomeric Ca^{2+} "stripes" [2] leading to the global rise of Ca^{2+} required for contraction. The close apposition of t-tubules or sarcolemma with sarcoplasmic reticulum (SR) at the dyadic junctions provides the structural bases for localized Ca^{2+}-cross signaling between the μ-domains associated with Ca^{2+} channels and ryanodine receptors (RyR) [2–4]. Calcium sparks originating from such junctions were first observed using fluo-3 in the line-scan mode of confocal imaging [1], and when imaged with 2-D rapid (240f/s) confocal microscopy, these elementary Ca^{2+} release events were seen to occur in sarcomeric patterns along z-lines [2]. The ability to resolve dyadic events was improved when 2–5 mM EGTA was included with Fluo-3 in the patch pipette solution to confine the diffusion of Ca^{2+}-bound fluorophore [5,6]. Use of line-scan mode of confocal imaging, without introduction of *silent* buffer EGTA, makes it more difficult to determine the actual size of the spontaneously occurring sparks [7]. The development of genetically engineered, targeted Ca^{2+}-biosensors with high fluorescence efficiency and rapid kinetics, potentially provide a better approach to probe the focal cardiac Ca^{2+}-signaling events than the diffusible cytosolic fluorescent dyes [8,9].

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[2] FK506 binding protein.
[3] I_{Ca}, L-type Ca^{2+} current.

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Toward this end, the RyR2-associated proteins (triadin, junctin and FKBP12.6) have been used to target genetically engineered Ca^{2+} probes to dyadic clefts to monitor the local release of Ca^{2+} from the SR through RyR2 [10,11]. Such studies have shed light both on junctional Ca^{2+} signaling and on persistent experimental limitations such as insufficient spatial resolution of imaging to focus on individual junctions, line scan *vs*. 2-D microscopy, correlation between junctional and cytosolic Ca^{2+} concentration, complications arising from over-expressed targeting probes, and, most importantly, the sensitivity, K_d, and speed of the engineered probe.

In this communication, we report on our attempts to target two high affinity Ca^{2+} biosensors to the RyRs of cardiac dyadic junctions by linking FKBP12.6 to the N-terminals of circularly permuted pericams. One of these probes, FKBP-YCaMP, uses an inverse pericam backbone [12] that responds to Ca^{2+} with decrease in fluorescence, a modification of which was previously used by us to target the mitochondrial matrix [13,14]. The other probe, FKBP-GCaMP6, using a GCaMP6 backbone [15], had much faster kinetics with strongly increasing fluorescence on exposure to Ca^{2+}.

We also used rapid 2-D TIRF microscopy to image simultaneously the signals generated by global cytosolic dye Fura-2 and two new RyR2-targeted dyadic probes to focus on subsarcolemmal layer of ~0.15 μm, giving us significantly higher z-resolution compared to confocal microscopy (~1 μm). Simultaneous recordings of cytosolic Ca^{2+} through the patch-pipette dialyzed Fura-2 provided the means of evaluating cytosolic *vs.* subsarcolemmal junctional Ca^{2+} signals in adult voltage-clamped rat ventricular cardiomyocytes in short-time cultures.

This experimental approach offers a number of potential benefits, but it also introduces experimental uncertainties. For instance, FKBP 12.6 and other members of the FKBP family [16], can bind to RyRs [17] and coordinate or stabilize Ca^{2+} release by homo-tetrameric RyR2 subunits [17,18], regulated perhaps by phosphorylation [19]. Thus, exogenous FKBP-anchored probe may inadvertently alter the activity of RyR2. Similarly, the TIRF technique by focusing on extremely thin surface layer maybe atypical for the interior of the cell measured confocally, or may show structural defects or motion artifacts depending on adhesion of cell membrane to the underlying glass surface.

Our data shows that FKBP-YCaMP and FKBP-GCaMP6 appeared to target the z-lines, co-localized competitively with RyR2-antibodies, and when overexpressed tended also to distribute between z-lines. TIRF images showed sarcomeric spacing as clearly as confocal sections and resolved the z-lines as punctate hot-spots of fluorescence suggesting individual dyadic junctions. Both probes yielded I_{Ca} and caffeine induced Ca^{2+} transients that were in general agreement with each other and those measured with Fura-2 (validating the TIRF technique), but had different kinetics. Spontaneous Ca^{2+} sparks measured with Fluo-4 AM were generally centered on subset of the fluorescence hot-spots of the expressed peptide probes, suggesting that most dyadic junctions were quiescent for extended periods of time. Fluo-4 sparks occurred more frequently in control than in cells infected either with FKBP-YCaMP or FKBP-GCaMP6, and were enhanced by isoproterenol, suggesting that the expressed probes contributed an element of FKBP-mediated regulation. FKBP-YCaMP failed to detect Ca^{2+} sparks or rapid spikes associated with rapid rise of dyadic Ca^{2+} concentrations possibly related to its slow kinetics, inverse response, and small dynamic range. FKBP-GCaMP6 probe, on the other hand, detected local I_{Ca}-triggered Ca^{2+} spikes that preceded slower cytosolic signals showing variability at different z-lines, and spontaneously occurring confined Ca^{2+} sparks, suggesting that genetically encoded targeted probes with fast on- and off-rate kinetics when combined with higher resolution of TIRF-microscopy bodes well for probing the Ca^{2+} nano-domains of dyadic clefts.

2. Materials and methods

2.1. Ethical approval

Animal protocols were approved and supervised by the Animal Care and Use Committees of the Medical University of South Carolina and University of South Carolina.

2.2. Isolation of adult rat ventricular myocytes, culture and adenoviral infection

Adult rat ventricular myocytes were isolated from male Sprague Dawley rats (200–250 g, 7–10 weeks old) using a standard enzymatic cell isolation method described previously [20]. Briefly, rats were deeply anaesthetized with isoflurane (3.0%), and hearts rapidly excised and perfused retrogradely at 7 ml min^{-1} through the aorta, first with Ca^{2+}-free solution containing (in mM): 137 NaCl, 5.4 potassium L-glutamate, 10 HEPES, 1 $MgCl_2$, 10 glucose, 0.6 Na_2HPO_4, 30 taurine, pH 7.2 at 37 °C, then with Ca^{2+}-free solution containing 0.4 mg ml^{-1} collagenase NB 8 (SERVA, Germany) and 0.16 mg ml^{-1} protease (Sigma) for 15–22 min, and finally with an enzyme-free solution containing 0.1 mM $CaCl_2$ for 8 min. The tissue was then cut into several pieces and gently agitated to dissociate the cells. The freshly isolated cells were plated on glass coverslips coated with extracellular matrix proteins (ECM gel from Engelbreth-Holm-Swarm murine sarcoma, Sigma) and left to equilibrate for 2 h. Attached cells were then cultured in M199 medium (Gibco) supplemented with (in mM): 20 albumin from bovine serum (BSA), 5 creatine, 2 L-carnitine, 5 taurine, 0.25 L-ascorbic acid and 1% insulin-transferrin-selenium-X (Corning) at 37 °C. Cardiac myocytes were then infected with adenovirus carrying the targeted genes at an m.o.i. of 500 and experiments were performed after 48 h in culture. For control experiments, cells from the same hearts underwent the same procedures except for the adenoviral infection.

2.3. Construction of recombinant adenovirus

Human FKBP12.6 cDNA was purchased from Open-Biosystems (Philadelphia) and was used as targeting moiety for two types of mutant circularly permuted pericams that both use *Xenopus* calmodulin as Ca^{2+} sensor. The cDNA for an inverse pericam [12] with YFP fluorescence that gets dimmer in the presence of Ca^{2+} [14] was developed from cDNA (M13-cpEYFP-CaM) kindly provided by Dr. Godfrey L. Smith (University of Glasgow, Scotland). The other pericam backbone (GCaMP6 [15]) incorporates GFP, responds to Ca^{2+} with an increase in fluorescence and was donated by Dr. Junichi Nakai (Saitama University, Japan). To construct expression plasmids of FKBP-YCaMP and FKBP-GCaMP6, DNA fragments flanked by specific restriction enzyme sites were amplified by polymerase chain reactions and were cloned in frame into dual-CCM(+) expression vector (Vector Biolab, United States). Complete DNA fragments amplified PCR were confirmed by DNA sequencing. The expression plasmids were used to produce adenovirus by Vector Biolab.

2.4. Immuno-labeling studies

Cardiac myocytes were fixed with 2% paraformaldehyde, blocked with 1% bovine serum albumin and 0.1% Triton X-100 in 10% PBS. The cells were incubated with anti-RyR monoclonal antibody (C3-33, Thermo scientific) for 24 h at 4 °C, washed with PBS and then incubated with Alexa Fluor-conjugated goat anti-rabbit IgG (Life Technologies) for 2 h at room temperature. The immunostaining was visualized using Leica SP5 confocal microscope at