Published in final edited form as:

Cell Calcium. 2017 September ; 66: 98–110. doi:10.1016/j.ceca.2017.07.003.

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

Sara Pahlavan^{a,1} and Marin Morad^a

^aCardiac Signaling Center of University of South Carolina, Clemson University and Medical University of South Carolina, Charleston, SC 29425 USA

Abstract

The details of cardiac Ca^{2+} signaling within the dyadic junction remain unclear because of limitations in rapid special 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, $K_d = 150\text{nM}$, or FKBP-GCaMP6, $K_d = 240\text{ nM}$) with rapid total internal reflectance fluorescence (TIRF) microscopy (resolution, $\sim 80\text{nm}$). The punctate z-line patterns of FKBP¹-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 ($\sim 20\%$) and too slow (2-3s) 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 $I_{\text{Ca}^{2+}}$, 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.

¹FK506 binding protein

² $I_{\text{Ca}^{2+}}$ L-type Ca^{2+} current

Corresponding author: Prof. Martin Morad, Ph.D., Cardiac Signaling Center, MUSC, Bioengineering Building, room 306, Charleston, SC 29425, U.S.A., Telephone: 843-876-2400, Fax: 843-876-2489, moradm@musc.edu.

¹Present address: Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Conflicts of interest: None.

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Keywords

Cardiac Ca^{2+} signaling; ryanodine receptor; FKBP; genetically engineered Ca^{2+} probe; Ca^{2+} sparks; dyadic Ca^{2+} signals

1. Introduction

During diastolic intervals, mammalian cardiomyocytes generate focal Ca^{2+} releases, “ Ca^{2+} sparks” [1], that are coordinated by Ica 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 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].

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 $\sim 0.15 \mu\text{m}$, giving us significantly higher z-resolution compared to confocal microscopy ($\sim 1 \mu\text{m}$). 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 hotspots 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 CaCl₂ 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 hours. 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 hours 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 Ca²⁺ sensor. The cDNA for an inverse pericam[12] with YFP fluorescence that gets dimmer in the presence of Ca²⁺[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 Ca²⁺ 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 hours at 4 °C, washed with PBS and then incubated with Alexa Fluor-conjugated goat anti-rabbit IgG (Life Technologies) for 2 hours at room temperature. The immuno-staining was visualized using Leica SP5 confocal microscope at 488 nm (FKBP-YCaMP and FKBP-GCaMP6) and 540 nm (Alexa-Fluor) excitation and 500-550 and >560 nm emission, respectively.

2.5. Studies of expression patterns using confocal and TIRF microscopy

Expression patterns of FKBP-YCaMP and FKBP-GCaMP6 were studied in fixed and live cells using Leica confocal (SP5) and TIRF (DMI6000 B) microscopes and were compared to those of other targeted peptide probes, mitycam[13] and PLN-YCaMP. The latter is a new construct that is similar to FKBP-YCaMP, but uses phospholamban to target SERCA. In other control TIRF experiments, cell adhesion was studied using membrane staining by di-4-ANNEPS.

2.6. *In situ* calibration of biosensors

Adult rat ventricular myocytes expressing FKBP-YCaMP or FKBP-GCaMP6 were permeabilized with 100 µg/ml saponin and incubated in Na⁺ and Ca²⁺-free bath solution containing (in mM): 5 BAPTA, 20 HEPES, 100 L-aspartic acid, 40 KCl, 1 MgCl₂, 2 maleic acid, 2 glutamic acid, 5 pyruvic acid, 0.5 KH₂PO₄, 5 µM thapsigargin and 5 µM ionomycin, pH 7.2 at room temperature. The fluorescence of FKBP-YCaMP and FKBP-GCaMP6 was recorded using TIRF and epi-fluorescence microscopy at 488nm excitation and 525-540 nm emission upon application of bath solutions containing different Ca²⁺ concentrations. Ca²⁺ concentrations were calculated based on BAPTA/Ca²⁺ concentrations and confirmed by the measurements of K₅Fura-2 fluorescent intensity in cell-free system. The relationship between Ca²⁺ concentration and the change in fluorescence, F , were obtained and fitted to the equation: $F = F_{\max} * [Ca^{2+}] / (K_d + [Ca^{2+}])$, where K_d is the dissociation constant.

2.7. TIRF microscopy for measurements of Ca²⁺ transients and sparks

Caffeine- or I_{Ca} -induced local Ca²⁺ releases were measured in cultured cells expressing FKBP-YCaMP and FKBP-GCaMP6 using total internal reflectance fluorescence (TIRF) microscopy (Leica DMI6000 B, Germany) at room temperature at 30 Hz. The Ca²⁺ current-induced releases were triggered in the whole cell configuration of the patch-clamp technique using a Dagan 8900 amplifier (Dagan Corp, Minneapolis). Membrane currents were digitized with a Digidata 1320A interface (Axon Instruments, Foster City, CA) with a sampling frequency of 10 KHz. Patch pipettes were filled with internal solution containing (in mM): 130 CsCl, 10 tetraethylammonium chloride, 5 NaCl, 10 HEPES, 5 MgATP, 10 glucose, 0.2 EGTA and 0.1 CaCl₂, pH 7.2. Interlaced measurements of global Ca²⁺ transients were obtained by adding 0.5 mM K₅Fura-2 and rapidly switching between laser beams exciting this Ca²⁺ probe at 405 nm and one of the peptide probes at 488 nm. Local Ca²⁺ transients were analyzed at regions of interests (ROI) selected close to the Z-lines. The average resting fluorescence intensity (F_0) was calculated from several frames measured immediately prior to voltage-clamp depolarization or caffeine applications. The amplitudes of the Ca²⁺ transients were quantified as F/F_0 , where F is the change in fluorescence.

Spontaneous diastolic Ca²⁺ sparks were measured with FKBP-GCaMP6, also in the two-dimensional TIRF mode, at a frequency (50-100 Hz) that was sufficient to resolve some features of their time courses, but did not allow interlaced measurements with Fura-2. For comparison, Fluo-4 AM (1 µM, 30 min incubation) was used to monitor Ca²⁺ sparks in control cells and cells expressing FKBP-YCaMP. The bath solution contained (in mM): 137 NaCl, 5.4 KCl, 10 HEPES, 10 glucose, 1 MgCl₂, 2 CaCl₂, pH 7.3.

2.8. Image processing and data analysis

Digital images were processed using custom-made program Con2j. To analyze Ca²⁺ sparks, a custom-made macro (sparks) was used in Con2j. Statistical analysis was performed in Prism (Graphpad) by using different tests (unpaired t-test, Mann-Whitney-Test, one-way ANOVA, Kruskal-Wallis-Test) depending on the results of the normality test (D'Agostino & Pearson omnibus normality test). Data are displayed as mean ± SEM. Asterisks indicate significant differences: p<0.05 (*), p<0.01 (**), p<0.001 (***).

3. Results

3.1. Targeting Ca^{2+} -sensitive biosensors to ryanodine receptors with FKBP12.6

To probe subsarcolemmal Ca^{2+} nano-domains in isolated ventricular myocytes, we constructed two virally-encoded high affinity biosensors that were targeted to the FKBP12.6- (calstabin) binding site of cardiac ryanodine receptors (RyR2) by incorporating human FKBP12.6 cDNA as shown in Fig. 1 A. Both probes are based on circularly permuted fluorescent protein that is flanked by M13 peptide at the N-terminal and calmodulin at the C-terminal to achieve Ca^{2+} sensitivity[21]. They differ with respect to the direction and magnitude of Ca^{2+} -induced fluorescence change as determined before adding FKBP12.6 at the N-terminal. In one probe, FKBP-YCaMP, we used an inverse pericam[12], that dims the yellow fluorescence by 20% in the presence of Ca^{2+} and was previously used to sense mitochondrial Ca^{2+} with a K_d of 220 nM [13, 22]). In the other newer construct, FKBP was linked to GCaMP6, which shows strongly increasing green fluorescence (~10 fold) in response to cytosolic Ca^{2+} (K_d = 160 nM [15, 23]).

Confocal images of Fig. 1 show distributions of FKBP-YCaMP in adult rat ventricular cells (Panels B and C) as compared to distributions found when using the same pericam backbone, but targeted to binding domains of phospholamban (PLN, Panel D) or the mitochondrial pre-sequence (MPS) of subunit VIII of cytochrome C oxidase (Panel E). Panel B shows a 3-D reconstruction that was rotated to show the RyR-targeted FKBP-YCaMP probe at well-defined lines corresponding to sharp z-lines with sarcomeric spacing (~1.8 μm). In a single confocal plane through the middle of the cells (Panel C) the regular z-line pattern of the probe is seen to extend from the cell surface (inset) to the interior except for interruptions by the nuclei (blue DAPI staining) and longitudinal darker bands suggestive of mitochondrial spaces. In cells with high expression of the FKBP-targeted probe, a fraction of molecules appears to be free-floating in the cytoplasm producing background fluorescence haze., the targeting of the FKBP to RyRs in the immediate vicinity of z lines was quite specific, producing no noticeable hotspots at other locations.

This interpretation is consistent with the fluorescence patterns generated by the mitochondrially targeted mitycam probe (panel E) showing a contrasting picture with bright longitudinal streaks with faint sarcomeric beading. In panel D, the PLN-linked probe is seen to form an intricate lace-like sarcomeric pattern of closely spaced bright dots at the z-lines and sharp longitudinal lines in between with some indication of stronger fluorescence half way between z-lines. The probe appeared also to stain the sarcolemma, and more intensely the nuclear envelope. The non-sarcomeric component of the distribution of the PLN-linked probe was unexpected.

In Fig. 2, the ryanodine receptors of ventricular cells infected with FKBP-YCaMP and FKBP-GCaMP6 were also fluorescence labeled by incubating the fixed cells with C3-33 monoclonal and Alexa Fluor antibodies, where green confocal images depict the distribution of the Ca^{2+} sensing peptides (488 nm excitation, 500-550 nm emission) and the red images the fluorescent antibody labeling (540 nm excitation, > 560 nm emission). Both fluorescence channels produced typical sarcomeric z-line patterns with a high degree of co-localization that is seen in the merged images as bright orange dots (see also Soeller *et al.* for high-

resolution studies of RyR distribution [24]). Inspection of cells with varying degree of expression and antibody labeling suggests that the Ca^{2+} probes and the antibodies bind competitively to RyRs, so that cells with low expression of FKBP-YCaMP or FKBP-GCaMP6 showed sharp z-lines for the antibody labeling (Fig. S1). Conversely, with higher expression of the Ca^{2+} probes, the antibody labeling became more diffuse producing a uniform red background fluorescence extending between z-lines and out to the cell surface (Fig. S2). Both the sarcomeric patterns and competitive binding confirm that the new probes bind to RyRs with a high degree of specificity.

In our Ca^{2+} signaling experiments we used TIRF imaging to focus on the subsarcolemmal space and achieved 0.1-0.2 μm depth of resolution that is considerably better than the $\sim 1 \mu\text{m}$ typical of confocal imaging resolution. It was important, therefore to test if the structure of the subsarcolemmal space, including the distribution of RyRs, is similar to that found in the interior of the cells. Figure 3 shows TIRF images obtained with 3 probes. Panel A shows that the FKBP-YCaMP probe is concentrated on the z-lines as in the confocal images of Figs. 1 and 2, but the longitudinal band with this pattern is now only 5-10 μm wide and fading to the sides. More significantly, the z-lines in the TIRF images, are now more punctate (arrow heads) suggesting that it may be possible to measure from RyR in couplons that are limited to the first $\sim 0.2 \mu\text{m}$ of the openings of t-tubules. Similarly, the mitochondrial staining with mitycam now shows longitudinal bands that are clearly resolved as beads with relatively narrow dark bands at the z-lines [25]. Thus, both of these peptide probes produce images that are more accurately resolved with TIRF than with confocal microscopy.

However, such improved images are only obtained when the cells make tight attachments with the underlying coverslip so that the evanescent illumination is not compromised by stray light emanating from impurities on the objective, in the immersion oil, in the glass cover slip and at the glass fluid interface. To examine such factors, we used rapid staining with di-4-ANNEPS to produce strongly fluorescent membrane-specific images (Fig. 3 D) in cells already expressing genetically engineered mitycam probe (Fig. 3C). The images show that unlike the banded mitycam images the di-4-ANNEPS image is relatively uniform and extends further away from the midline of the cell where it terminates at a well-defined, but irregular boundary. These staining patterns suggest that the contact between the sarcolemma and the underlying glass cover slip is tight and mechanically stable over extended areas, even when the cells are subjected to pressurized puffs of solutions. But the data also suggests that the membrane at the periphery of these contact areas may be pulled away from the underlying contractile and Ca^{2+} -signaling machinery.

To measure the Ca^{2+} sensitivity of FKBP-linked probes *in situ* in saponin-permeabilized adult rat ventricular myocytes, the myocytes were subjected to different BAPTA-buffered Ca^{2+} concentrations at pH 7.2. The calculated K_d values were $\sim 150 \text{ nM}$ for FKBP-YCaMP (Fig. 3E) and 240 nM for FKBP-GCaMP6 (Data not shown). Similar values were reported for the parent compounds ($K_d = 216 \text{ nM}$ at pH 7 for mitycam [13] and 160 nM for GCaMP6 [15]). Thus, the new FKBP-targeted Ca^{2+} sensors appear to bind with high specificity and saturation kinetics to RyR at the z-lines when expressed in adult rat ventricular myocytes, producing *in situ* similar signals as those of untargeted probes.

3.2. Recordings of cellular Ca^{2+} transients with FKBP-YCaMP

The properties of FKBP-YCaMP as a targeted Ca^{2+} sensor in rat ventricular cardiomyocytes were compared to those of Fura-2 by performing sequential interlaced TIRF imaging, alternating between λ_{ex} 488 and 405 nm. At these wavelengths of excitation, both probes respond to Ca^{2+} with a decrease in fluorescence (Fig. 4). Ca^{2+} releases from the SR were triggered either by 100 ms depolarizing pulses from -50 to 0 mV, activating I_{Ca} (Fig. 4 A, B) or by 2 sec long puffs of 3 mM caffeine (Fig. 4 C, D). In contrast-enhanced images of FKBP-YCaMP (Fig. 4A), the sarcomeric hotspots of fluorescence were seen to fade from purple and red into green, yellow and blue during I_{Ca} -triggered Ca^{2+} releases that were repeated at 5 s intervals. The time course of the fluorescence intensity showed no significant difference between selected color-coded regions of interest (ROI, Fig. 4 B): The relatively rapid (0.59 ± 0.03 s, time to peak) fall of fluorescence signal was followed by a much slower relaxation of the signal back to baseline level that was often incomplete within the 5s inter-pulse periods. The Fura-2 images differed from those of FKBP-YCaMP by being faster (time-to-peak, 0.34 ± 0.03 s), more uniform throughout the cells, (see ratiometric TIRF images before, Fig. 4C top, and during, Fig. 4C bottom, of a caffeine-triggered Ca^{2+} release pulse (Fig. 4 D).

Using FKBP-YCaMP, we failed to detect any spontaneous Ca^{2+} sparks during the diastolic periods, probably because the probe fails to achieve sufficient signal to noise ratio and temporal resolution to detect such localized Ca^{2+} -dependent dimming events. Similarly, we failed to detect reliable dimming Ca^{2+} sparks even when using Fura-2 dye at 405 nm. Nevertheless, imaging with FKBP-YCaMP produced 2 types of results relevant to spark formation and Ca^{2+} signaling in general: First, when the FKBP-YCaMP-infected cells were imaged using Fluo-4 (both excited at 488 nm), the FKBP-YCaMP probe, as expected, produced the phenotypic sarcomeric fluorescence images (Fig. 5 A, F_0) while Fluo-4 revealed Ca^{2+} sparks (F) that were often, but not always, centered on the hot spots marking the distribution of the FKBP-linked probe. It was interesting that in recording periods lasting 15 or 30 s, although most sites were completely quiescent, producing a few isolated sparks, a few sites that did not show intense levels of binding to the RyR-targeted probe (red and orange ROIs and traces in Fig. 5 C; supplementary Figs. S3 and S4) fired repeatedly, consistent with the possibility that FKBP probe binding was producing a biological inhibitory effect at the majority of the release sites. Second, the expression of FKBP-YCaMP might be used as a tool to evaluate the role of FKBP as a modulator of RyRs.

To test the latter possibility, we compared the unitary properties of Ca^{2+} sparks measured by Fluo-4 both in control and FKBP-YCaMP expressing myocytes, and found that in the cells infected with the targeted probe, Ca^{2+} sparks occurred less frequently (3.84 ± 0.98 vs. 7.13 ± 1.71 per cell $^{-1}$.s $^{-1}$, Fig. 5 E), and were of smaller amplitudes (Fig. 5 G and H), but of same duration, 5F (~ 20 ms based on recordings at 70 frames s $^{-1}$). This finding is consistent with reports that overexpression of FKBP12.6 silences spontaneous RyR2 openings and decreases their mean open time [26, 27].

3.3. Comparison of Ca^{2+} signals measured with FKBP-YCaMP and FKBP-GCaMP6

The slow kinetics, small dynamic range, and Ca^{2+} -induced dimming fluorescence of the FKBP-YCaMP probe led us to design another probe targeted to RyR2 with faster kinetics and increasing fluorescence intensity on binding Ca^{2+} . As shown above, this construct (FKBP-GCaMP6, Fig. 1 A) was targeted to RyR2 as effectively as the inverse FKBP-YCaMP probe, and had a similar K_d (240 nM) based on *in situ* measurements where Fura-2 ($K_d = 220$ nM) served as reference (Data not shown).

Figure 6 compares the Ca^{2+} signals that were recorded with the two probes in cells where Ca^{2+} releases were activated by I_{Ca} . In both cases the fluorescence responses developed along sarcomeric lines as defined by the baseline fluorescence (Panels A and D). Compared to the fluorescence decreases measured with FKBP-YCaMP (panel E), the FKBP-GCaMP6 probe produced fluorescence increases that developed faster (0.08 ± 0.01 s vs. 0.59 ± 0.03 s), had an initial spike-like phase, and decayed in two phases. Using regions of interest corresponding to successive segments of z-lines (~ 5 μm long), we found that peak amplitudes of Ca^{2+} transients showed greater variability from one z-line to the next when measured with FKBP-GCaMP6 than with FKBP-YCaMP (Panels B and E, *cf.* colored bars and traces). The consistency of this finding is illustrated in panels C and F, which show pooled data from 5 cells (different symbols) expressing either FKBP-YCaMP or FKBP-GCaMP6. The faster kinetics, rapidly decaying initial spike, and variability of the fluorescence signals measured with FKBP-GCaMP6 all suggest that this probe primarily responds to focal I_{Ca} -induced dyadic Ca^{2+} releases and only secondarily to the integrated, more uniform elevation of cytosolic Ca^{2+} . This implies that, for the first time, it is possible not only to target a Ca^{2+} probe to dyadic clefts, but also to achieve sufficient sensitivity and on-rate to catch a significant number of the released calcium ions that are in transit through this confined dyadic space.

3.4. Ca^{2+} sparks as measured with GCaMP6-FKBP and Fluo-4

Figure 7 compares the TIRF images (100Hz) of spontaneous Ca^{2+} sparks measured in two different intact cells, one infected with the FKBP-targeted probe (Fig. 7A) and the other incubated in Fluo-4AM (Fig. 7C). The FKBP-GCaMP6 Ca^{2+} sparks appeared centered on “dyadic” hotspots base-line image (F_0) that were confined in successive frames and could be resolved as distinct events even when occurring within ~ 1 - 1.5 μm of each other (Fig. 7E). In sharp contrast Fluo-4 Ca^{2+} sparks were more diffuse, often spreading over a much larger distance that exceeded the spacing of hot-spots of RyR2 labeling between adjacent z-lines (Fig. 7C & F). Comparative data of the unitary properties of the Ca^{2+} sparks measured with the two probes, Figure 7B and D, shows that although the rise time of sparks measured with the two probes was not substantially different ($t_{1/2\text{max}}$: 17.2 ± 1.4 ms in FKBP-GCaMP6 vs. 15 ± 0.7 ms for Fluo-4), the GCaMP6-FKBP sparks were somewhat smaller in amplitude and occurred less frequently. The predominant difference in the spark properties, measured with the two probes, was in size and frequency of occurrence of the unitary Ca^{2+} release events (Fig. 7D).

3.5. β -adrenergic effects on FKBP-GCaMP6 Ca^{2+} signals

It is well established that β -adrenergic agonists enhance cardiac contraction by mechanisms that include PKA-induced phosphorylation of L-type Ca^{2+} channels [28], RyR2 [29], phospholamban, and contractile myofilaments [30]. The phosphorylation increases both the rate of Ca^{2+} release by RyR2 and Ca^{2+} uptake by SERCA2a. It is somewhat more controversial whether there is also a direct functional effect of β -adrenergic agonists on RyR2, mediated by unbinding of FKBP12.6 from RyR2 [31-33].

We examined whether FKBP-GCaMP6 expression might also exert a regulatory effect on RyR2 similar to those of FKBP-YCaMP (Fig. 5). Using matched cells from the same hearts we found that the frequency of spontaneous Fluo-4 Ca^{2+} sparks was significantly smaller in FKBP-GCaMP6-expressing cells compared to those measured in control myocytes ($0.91 \pm 0.17 \text{ s}^{-1}$ vs. $2.04 \pm 0.3 \text{ per cell}^{-1} \cdot \text{s}^{-1}$ in Control), similar to the finding in cells that expressed FKBP-YCaMP (Fig. 5). Interestingly in FKBP-GCaMP6 infected cells, with lower baseline sparking frequency, isoproterenol markedly enhanced the frequency of spontaneously occurring sparks (Fig. 8B), while having little or no effect on the frequency of spontaneously occurring sparks in control cells. These findings support the possibility that PKA phosphorylation may alter FKBP 12.6 binding of RyR2 [18, 19, 34].

4. Discussion

To detect Ca^{2+} release signals within the dyadic junctions, rat ventricular cells were infected with two newly developed genetically engineered fluorescent Ca^{2+} probes targeted to RyR2 and their subcellular staining patterns imaged using TIRF microscopy ($\sim 100\text{nm}$ resolution). One probe, FKBP-GCaMP6, appeared to have the appropriate Ca^{2+} -sensitivity and kinetics to monitor the unitary “ Ca^{2+} sparks”, and detect focal I_{Ca} -triggered Ca^{2+} releases. FKBP was chosen as the binding target on RyRs, because its binding site appears to be in the vicinity of the Ca^{2+} release pore, increasing the probability of detecting the Ca^{2+} transiting through dyadic clefts. The use of FKBP binding site, however, has the caveat that it could modify the Ca^{2+} release activity of RyR2. We therefore validated this approach by comparing the Ca^{2+} signals measured by our new probes with Fluo-4 and Fura-2 dialyzed through the patch pipette. Our data suggests that FKBP-GCaMP6 probe binds predominantly to RyR2 within the dyadic clefts where it detects rapid local Ca^{2+} releases, measured as fluorescence spikes that are centered on punctate hotspots of baseline fluorescence along z-lines. Such spikes develop from one frame to the next (30-100 Hz) and decay with a time constant of $\sim 100 \text{ ms}$ that reflects the off-kinetics of the probe. We have resolved these spikes as local events both in the form of spontaneous Ca^{2+} sparks or when they are triggered synchronously at most sites by I_{Ca} . A fraction of probe molecules appears also to be distributed in the global cytosolic space where they detect longer lasting I_{Ca} and caffeine-induced Ca^{2+} releases. Such delayed signals developed slowly, were generally larger, and lacked the initial fluorescence spikes. In addition, some Ca^{2+} sparks were preceded by local nearby fluorescence transients with somewhat wandering fluorescence signals occurring repeatedly at sites outside the punctate z-line pattern of RyR2 staining. Comparison of frequency of Ca^{2+} sparks in FKBP-GCaMP6-expressing and control cells,

suggest that the probe exerts stabilizing effects that have been associated with FKBP and β -adrenergic induced phosphorylation.

4.1. Imaging of targeted probes: TIRF vs. confocal microscopy

TIRF microscopy is generally used to monitor structures and events occurring in the vicinity of the surface membrane such as exocytosis, cellular internalization, micro-tubular rearrangements, and movements of single fluorescence-tagged proteins [35]. The resolution of this technique has already been demonstrated by measurements of Ca^{2+} signaling events such as Ca^{2+} sparks, Ca^{2+} fluxes through single L-type Ca^{2+} channels, Ca^{2+} signaling between the surface membrane and adjacent mitochondria. The present study is the first to use TIRF microscopy in conjunction with genetically engineered Ca^{2+} probes to focus on Ca^{2+} signaling within dyadic junction of adult ventricular cardiomyocyte. It was important therefore to ascertain whether this dual targeting approach provides real advantages or is undermined by a new set of technical pitfalls.

Figures 1-3 demonstrate that the new FKBP-linked probes are accurately targeted to RyRs. Although confocal 3-D reconstruction showed that the probes were located in the immediate vicinity of z-lines (Fig. 1B), TIRF microscopy proved superior in resolving these z-lines into a punctate pattern (TIRF: Figs. 3A, 4A, 5A & B, 6A & D, 7A & E, confocal: Figs. 1C, and 2) suggestive of individual dyadic junctions [5]. Similar improvements were also seen with the mitycam probe where TIRF microscopy resolved longitudinal mitochondrial bands into bean-shaped elongated sarcomeric segments (TIRF: Fig. 3B vs. confocal: Fig. 1E). Since the resolution in the horizontal (x-y) plane is comparable, the improvement must be attributed to the thinness (0.1-0.2 μm) of the exponentially decaying evanescent field of TIRF-illumination versus the point spread function of confocal instruments, typically $\sim 1\mu\text{m}$ in the vertical (z) direction, in the absence of “super resolution” refinements [35]. The improved resolution also testifies that the immobilized tight adhesions developing between the cells and the underlying glass coverslips (Fig. 3D), do not generate excessive stray light in the form of “TIRF shadows” [36]. Similarly, we found that the regular sarcomeric patterns of the isolated adult cardiomyocytes during short time culture (2-5 days) remained undisturbed in both intact (Fig. 1), or adhering (Fig. 3) surfaces of the cell. Our FKBP-linked Ca^{2+} biosensors not only co-localized with the staining pattern of fluorescent RyR2 antibodies (Fig. 2), but also appeared to have a free-floating inter-myofibrillar component, suggestive of saturation when overexpressed or displaced by the RyR2 antibodies. Unlike the RyR2 antibodies (Fig. 2) or the probe that was targeted to SERCA/PLB complex (Fig. 1D), the FKBP-targeted probes showed no membrane or other non-specific staining patterns. In particular, the FKBP-linked probes neither adhered to surface membrane, nor did they show any indication of subsarcolemmal peripheral couplings outside z-lines, as found in atrial cells [37] or association with mitochondria [38] from which they are clearly excluded (Cf. 1C and 1E). Thus, the FKBP-YCaMP and FKBP-GCaMP6 probes appear to bind with high specificity to RyR2 in dyadic junctions that can be clearly resolved with TIRF microscopy.

4.2. Detection of cytosolic and dyadic Ca^{2+} concentrations with FKBP-YCaMP and FKBP-GCaMP6

The K_d 's of our two probes did not significantly change on addition of FKBP12.6 targeting moiety. In permeabilized cells we measured a K_d of $\sim 150\text{ nM}$ for FKBP-YCaMP (Fig. 3E) as compared to 216 nM when the same pericam backbone was targeted to mitochondria [13]. Similar *in situ* measurements produced a $K_d = 240\text{ nM}$ for FKBP-GCaMP6 compared to 160 nM for untargeted GCaMP6[15]. In other attempts to target the dyadic cleft space, Despa *et al.* [11] and Shang *et al.* [10] found that their low affinity pericam was influenced by the cellular environment, but not by the addition of FKBP12.6 to its carboxyl-end. They report that GCaMP2.2-low effectively measured the global calcium $[\text{Ca}^{2+}]_{\text{Bulk}}$ with peak values of $560 \pm 73\text{ nmol/L}$ as compared to ~ 2 -fold higher values for cleft Calcium ($[\text{Ca}^{2+}]_{\text{Cleft}}$) measured with GCaMP2.2low-FKBP. Nevertheless, both values were significantly smaller compared to what has been previously estimated (10 to $15\text{ }\mu\text{M/L}$) [11]. Shang *et al.*, on the other hand, found a 4-times faster off-rate and lower K_d when triadin or junctin were added to the C-terminus of GCaMP6f to anchor it to RyR2.

As shown above, FKBP-GCaMP6 (Fig. 1 A) was targeted to RyR2 as effectively as the inverse FKBP-YCaMP probe, and it had a similar K_d (240 nM) based on *in situ* measurements where Fura-2 ($K_d = 220\text{ nM}$) served as reference (Data not shown). The calculated K_d values were $\sim 150\text{ nM}$ for FKBP-YCaMP (Fig. 3E) and 240 nM for FKBP-GCaMP6 (Data not shown). Similar values have been measured for the parent compounds ($K_d = 216\text{ nm}$ at pH 7 for mitycam[13] and 160 nM for GCaMP6[15]).

The sensitivity and kinetics of GCaMP6-FKBP appears to be appropriate for this novel Ca^{2+} biosensor to provide detailed information on spatial and temporal properties of Ca^{2+} release at the dyadic clefts. While the K_d of both probes were in few hundred nano molar range, they did resolve the dyadic Ca^{2+} release events in micro-molar range triggered by membrane depolarizations (Fig. 4B). Accordingly, Ca^{2+} sparks were found to be much smaller in volume as they remained confined to z-lines. Consistent with these findings, a similar probe, GCaMP6f, targeted to triadin or junctin was also reported to monitor individual Ca^{2+} nano-sparks 50 times smaller in volume compared to conventional Ca^{2+} sparks measured with diffusible dyes like Fluo-4 [10]. Furthermore, a low affinity GCaMP2.2, either targeted to FKBP12.6 or untargeted, could demonstrate diastolic gradient and EC-coupling induced dynamic differences of $[\text{Ca}^{2+}]_{\text{Cleft}}$ and $[\text{Ca}^{2+}]_{\text{bulk}}$, respectively [11]. Using the higher resolution of TIRF imaging and our two targeted probes to RyR2, we found that only GCaMP6 had the high sensitivity to detect Ca^{2+} sparks, and a Ca^{2+} on-rate kinetics that were fast enough to track the rapid Ca^{2+} release events of RyRs. Consistent with these findings, GCaMP6 has been reported to detect fast neuronal spikes [15].

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—Despite being highly efficient, GCaMP6-FKBP detected sparks with markedly lower frequency compared to conventional fluo-4. The stabilizing effect of FKBP12.6 on the RyR2 has been already reported by others, where the overexpression of FKBP12.6 in cardiomyocytes significantly reduced diastolic RyR2 leakage [39], and where spark frequency was reduced in transgenic mice overexpressing cardiac FKBP12.6 [27]. This

reduction in release events has been proposed to occur by stabilizing RyR2 in its closed state during diastole [40]. Because we introduced exogenous FKBP12.6 in cardiomyocytes by expressing GCaMP6-FKBP, it is likely that our cells would resemble those overexpressing FKBP12.6. Indeed, our results are consistent with the hypothesis that FKBP12.6 stabilizes RyR2 by reducing the frequency of occurrence of spontaneous sparks, rendering β -adrenergic stimulation more effective in enhancing the spark frequency in ventricular myocytes expressing GCaMP6-FKBP, Fig. 7.

4.3. RyR2-targeted probes: their potential and pitfall

The results presented here and those of Despa *et al.*[11] and Shang *et al.*[10] show that it is not only possible to measure dyadic Ca^{2+} release signals in cardiac cells with genetically engineered fluorescent probes targeted to RyR2, but also suggest that additional improvements may be necessary before this technique can be routinely applied to evaluate EC-coupling dysfunction encountered in cardiac hypertrophy and inherited cardiac diseases.

One pitfall of this approach is the likelihood of overexpression of the probes resulting in saturation binding thus generating in addition a cytosolic signal. This additional cytosolic signal may not be without interest, but would be more useful if it were clearly distinguishable from the dyadic component – not only by its location but also by its spectral properties as illustrated here by the addition of Fura-2 with near UV excitation. One approach to resolve this difficulty is to examine cells not for their overall fluorescence intensity, but for clear punctate patterns with minimal fluorescence between z-lines. Another approach would be to engineer the probe to generate Ca^{2+} -dependent fluorescence only when bound to its target or to use a split probe [41] that generates a signal when its two halves are bound to dual targets on the RyRs.

A more fundamental problem arises from marginal nature of the probes with respect to their sensitivity and kinetics. In this respect, it is essential that the effective on-rate (k_{on}) of probes should be sufficient to catch a significant fraction of the released calcium ions that are in transit through the dyadic cleft on their way to activate the contractile filaments in the global cytosolic space. A rough estimate of this ability may be obtained as $k_{\text{on}} = k_{\text{off}}/K_d$, where the steady state K_d is measured in permeabilized cells (Fig. 3) and $k_{\text{off}} = 1/\tau_{\text{off}}$ is the fastest measured rate of fluorescence fading as with local Ca^{2+} release and non-diffusible probe. As illustrated in table S5, available numbers suggest that k_{on} of the genetically engineered probes generally falls 2-3 orders of magnitude below those of Fura-2, Fluo-4 and BAPTA which are still about one order of magnitude below the “diffusion limit” of biological reactions, $\sim 10^9 \text{ M}^{-1}\text{s}^{-1}$. It should be noted that k_{on} of our high affinity probe is similar to that of the lower affinity probe used by Shang *et al.*[10]. similarly, the engineering of low affinity probes to measure the suspected higher Ca^{2+} concentrations in the cleft space is not warranted unless the duration of the Ca^{2+} release exceeds the equilibration time of the probe. In this instance, the probe would be counterproductive if Ca^{2+} measurement is accomplished by decreasing k_{on} [11] instead of increasing k_{off} . While these simple, first-order considerations provides some guidelines for evaluating the ongoing efforts to produce new faster and brighter probes [42], fine tuning is likely to require direct measurements of Ca^{2+} -induced fluorescence rise times (*e.g.* using caged Ca^{2+} [43]) and consideration of higher

order kinetics where the calmodulin, depending on EF-hand mutations, binds as many as 4 Calcium ions and undergoes conformational changes. Considering the observed speed of Ca^{2+} -mediated inactivation of I_{Ca} and activation of fast twitch fibers, it is not unreasonable to hope that such efforts may ultimately produce Ca^{2+} probes that approach the diffusion limit, given sufficient signal for rapid detection of Ca^{2+} activity within the dyadic clefts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are thankful to Prof. Nakai and Dr. Ohkura (Saitama University, Japan) for kindly providing GCaMP6 cDNA. We would like to thank Cassandra Clift for editing of the Manuscript.

Funding: This work was supported by a grant from NIH RO1 HL16152.

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Highlights

- We generated FKBP-YCaMP and FKBP-GCaMP6 as genetically engineered RyR2-targeted probes
- FKBP-YCaMP signals were too small (~20%) and too slow (2-3s) to detect Ca^{2+} - sparks
- FKBP-GCaMP6 had sufficient on-rate to monitor dyadic Ca^{2+} sparks in the Z-lines
- ISO did not dissociate FKBP-GCaMP6 from z-lines; enhanced Ca^{2+} spark frequency

Figure 1. Two new fluorescent probes targeted to ryanodine receptors with FKBP12.6 (A) and confocal images of their distribution in adult rat ventricular cells (B-C) as compared to probes targeted to SERCA (D) and mitochondria (E)

A: Schematic cDNA sequences for two new probes, FKBP-YCaMP and FKBP-GCaMP6, where FKBP12.6 is linked to the N terminal of previously used constructs (Based on [13, 15, 22, 23]). **B:** Contrast-enhanced 3-D reconstruction based on z-stack showing targeting of FKBP-YCaMP to z-lines. **C-E:** Comparison confocal images showing the distribution of fluorescent probes targeted with FKBP to RyR (**C:** FKBP-YCaMP), phospholamban to SERCA (**D:** PLN-YCaMP) and the mitochondrial targeting sequence of subunit VIII of human cytochrome c oxidase (**E:** Mitycam [13, 14]). The confocal plane was adjusted to the midline of the cells where blue DAPI staining shows the locations of nuclei in the fixed cells in panels **C** and **D**, but not in the live cell in panel **E**. Inset panels show enlargements of boxed areas.

Figure 2. Colocalization of FKBP-YCaMP (A) and FKBP-GCaMP6 (B) with immunolabeled RyR (RyR₂ antibody)

Figure 3. TIRF imaging clearly resolved sarcomeric fluorescence patterns of fluorescent Ca^{2+} probes targeted to RyR (A) and mitochondria (B) in strongly adhering ventricular cardiomyocytes (C, D)

E: Changes in fluorescence intensity vs. $[\text{Ca}^{2+}]$ in permeabilized, FKBP-YCaMP expressing cell.

Figure 4. Comparison of cellular Ca^{2+} transients measured simultaneously with FKBP-YCaMP and Fura-2

A: Images showing decreases in the local intensity of FKBP-YCaMP measured on activation of I_{Ca} by depolarization from -50 mV to 0 mV and selected regions of interest (ROI). **B:** Time course of fluorescence changes in ROIs and (expanded) current traces indicating timing of activation of I_{Ca} . **C:** Images of Fura-2 fluorescence in cardiomyocyte showing uniform decreases in intensity during caffeine triggered Ca^{2+} release. **D:** Interlaced recording of Ca^{2+} signals measured with FKBP-YCaMP and Fura-2 showing slower rise (histogram) and decay times for the genetically engineered probe.

Figure 5. Ca^{2+} sparks measured with fluo-4 in cells with and without expression of FKBP-YCaMP

A: Baseline fluorescence of FKBP-YCaMP and Fluo-4 both excited at 488 nm. **B:** Contrast enhanced image showing targeting of FKBP-YCaMP to z-lines. **C:** Detail of panel B with color-coded regions of interest (ROI) and numbered sample frames showing examples of infrequent (top) or regularly recurring Fluo-4 Ca^{2+} sparks (bottom, red and orange ROIs). **D:** Time course of fluorescence intensity measured at 30 Hz in ROIs with details shown on expanded time scale in panel H. Panels **E**, **F** and **G**: Comparison of the frequency (**E**), duration (**F**), from recordings at ~ 100 Hz and amplitude (**G**) of Fluo-4 Ca^{2+} sparks measured with Fluo-4 in control cells and in cells expressing FKBP-YCaMP.

Figure 6. Comparison of local I_{Ca} -activated Ca^{2+} signals measured with FKBP-GCaMP6 (A, B, C) and FKBP-YCaMP (D, E, F)

A, D: Contrast enhanced images of background fluorescence measured with FKBP-GCaMP6 (A) and FKBP-YCaMP (D) and color-coded regions of interest (ROI) defined along sarcomeric z-line ridges. **B, E:** Time course of I_{Ca} -activated fluorescence changes ($\Delta F/F_0$) measured in 3 selected ROIs, preceded by columns comparing their peak amplitude. **C, F:** Variability in $\Delta F/F_0$ measured at a sequence of sarcomeric lines as defined in panes A and D. The symbols in panels C and D correspond to 5 different cells for each of the FKBP-linked probes.

Figure 7. Properties of Ca^{2+} sparks measured with GCaMP6-FKBP (A) and Fluo-4 (C)
A, E: Background fluorescence and sample frames with Ca^{2+} sparks recorded in different cells. B: Time course of fluorescence intensity at the location showing Ca^{2+} sparks in panels A (top, FKBP-GCaMP6) and C (bottom, Fluo-4). D: Average values of Ca^{2+} spark parameters (amplitude, frequency of occurrence, rise time) measured with FKBP-GCaMP6 (green columns) and Fluo-4 (black columns). E, F: Two sequences of fluorescence images showing background fluorescence (F_0 with, E, and without, F, contrast enhancement) followed by sequences of F-images recorded at 100 Hz.

Figure 8. Effect of isoproterenol on number of Ca²⁺ sparks measured with Fluo-4 in control cells and in cells expressing FKBP-GCaMP6

A: Sample frames selected to illustrate the increase in the number of Ca²⁺ sparks that is produced by 1 μ M isoproterenol in cells expressing FKBP-GCaMP6. B: Histogram showing average values of Ca²⁺ spark frequency.