Cardiac Myocyte Z-Line Calmodulin Is Mainly RyR2-Bound, and Reduction Is Arrhythmogenic and Occurs in Heart Failure

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Rationale: Calmodulin (CaM) associates with cardiac ryanodine receptor type-2 (RyR2) as an important regulator. Defective CaM–RyR2 interaction may occur in heart failure, cardiac hypertrophy, and catecholaminergic polymorphic ventricular tachycardia. However, the in situ binding properties for CaM–RyR2 are unknown.

Objective: We sought to measure the in situ binding affinity and kinetics for CaM–RyR2 in normal and heart failure ventricular myocytes, estimate the percentage of Z-line–localized CaM that is RyR2-bound, and test cellular function of defective CaM–RyR2 interaction.

Methods and Results: Using fluorescence resonance energy transfer in permeabilized myocytes, we specifically resolved RyR2-bound CaM from other potential binding targets and measured CaM–RyR2 binding affinity in situ (K_d=10–20 nmol/L). Using RyR2^ADAD/mice, in which half of the CaM–RyR2 binding is suppressed, we estimated that >90% of Z-line CaM is RyR2-bound. Functional tests indicated a higher propensity for Ca^2+ wave production and stress-induced ventricular arrhythmia in RyR2^ADAD/mice. In a post–myocardial infarction rat heart failure model, we detected a decrease in the CaM–RyR2 binding affinity (K_d=51 nmol/L; =3-fold increase) and unaltered RyR2 affinity for the FK506-binding protein FKBP12.6 (K_d=0.8 nmol/L).

Conclusions: CaM binds to RyR2 with high affinity in cardiac myocytes. Physiologically, CaM is bound to >70% of RyR2 monomers and inhibits sarcoplasmic reticulum Ca^2+ release. RyR2 is the major binding site for CaM along the Z-line in cardiomyocytes, and dissociating CaM from RyR2 can cause severe ventricular arrhythmia. In heart failure, RyR2 shows decreased CaM affinity, but unaltered FKBP 12.6 affinity. (Circ Res. 2014;114:295-306.)

Key Words: arrhythmias, cardiac fluorescence resonance energy transfer heart failure ryanodine receptor calcium release channel

Approximately 50% of heart failure (HF) patients die of ventricular arrhythmia and sudden cardiac death. It is known that ryanodine receptor type-2 (RyR2)-mediated Ca^2+ leak from the sarcoplasmic reticulum (SR) during diastole can activate inward current via Na/Ca exchange and evoke delayed afterdepolarizations. In HF, there is enhanced diastolic SR Ca^2+ leak via RyR2 and other electrophysiological changes that greatly enhance the propensity for delayed afterdepolarizations and triggered cardiac arrhythmias, a leading cause of ventricular tachyarrhythmia and sudden cardiac death. Thus, RyR2 has emerged as a potential therapeutic target for treating HF and arrhythmia. Stabilizing RyR2 and preventing abnormal Ca^2+ leak (without affecting normal excitation–contraction coupling) may be a valid therapeutic approach.

Calmodulin (CaM) is an important RyR2 regulator, but has multiple cellular targets. CaM has 2 pairs of globular Ca^2+-binding E-F hand domains connected by a flexible linker. At high [Ca]^2+, Ca^2+ binds cooperatively to CaM, inducing a conformational change that translates intracellular [Ca]^2+ signals to diverse processes via many targets, including myosin light chain kinase, calcineurin, nitric oxide synthase, phosphodiesterase, adenylyl cyclase, Ca^2+/CaM-dependent kinase (CaMK), Ca^2+-activated potassium channels, r-type Ca^2+ channels (LTCCs), and RyRs.

CaM binds to RyR2 stoichiometrically (4 CaMs per tetrameric RyR2), and amino acids 3583 to 3603 on RyR2 are essential for this interaction. CaM inhibits RyR2 opening at all [Ca]^2+ and, as such, may be a critical regulator of SR Ca^2+ release. Cryo-EM–based 3-dimensional reconstruction studies showed that Ca^2+-free CaM (apoCaM) binds in the RyR cytosolic domain 3, within 60Å to 70Å from FK506-binding protein 12.6 (FKBP12.6) bound on the same RyR face.
and we have demonstrated that fluorescence resonance energy transfer (FRET) can be measured between these partners bound to either RyR1 or RyR2. FKBP12.6 was reported to bind RyR2 tightly as an important regulator in RyR2 gating, but aspects of the FKBP12.6–RyR2 interaction and function remain controversial. It was also reported that CaM could bind RyR2 in the closed state during diastole and facilitate stabilization of RyR2 in the closed state during diastole and facilitate stabilization of RyR2 and to cause severe hypertrophic cardiomyopathy and early death in animals. In nonischemic HF animal models, CaM binding to RyR2 was decreased. A recent study indicates that defective CaM binding to RyR2 is also involved in catecholaminergic polymorphic ventricular tachycardia (CPVT)-associated RyR2 dysfunction. So, the CaM–RyR2 interaction may be critical for arrhythmias and HF pathogenesis.

Here, we used FRET to resolve RyR-bound CaM from other CaM targets and, in the native myocyte environment, to characterize CaM–RyR2 interaction properties for normal and HF cardiomyocytes. Knock-in (KI) heterozygous mice (RyR2ADA/+), with genetically disrupted CaM–RyR2 association (W3587A/L3591D/F3603A, denoted ADA), were used to estimate what percentage of Z-line–bound CaM is RyR2-bound and to test for increased arrhythmia susceptibility in both myocytes and intact animals under acute stress. This would be analogous to what is seen in CPVT, which is linked to RyR2 mutations. This ADA mutant RyR2 is not expected to change CaM effects at other CaM targets. Human mutations in CaM have recently been associated with CPVT, and we believe that these RyR2ADA/+ mice represent a potential model for CPVT, which is linked to RyR2 mutations and CaM dysfunction.26

Methods

Ventricular myocytes were isolated as previously described from hearts of wild-type (WT) or RyR2ADA/+ K1 mice and Sprague–Dawley rats (control and 12 weeks after HF induced by coronary ligation). FKBP12.6 and CaM were labeled with Alexa Fluor 488 and 568 as described, which bind to and regulate RyRs in SR similar to WT proteins. Some myocytes were saponin-permeabilized and bathed in physiological internal solutions. Fluorescent FKBP12.6 and CaM (F-FKBPs and F-CaM) and 25 μmol/L Fluo-4 (to measure [Ca2+]) were used with confocal imaging. EGos were recorded in RyR2ADA/+ and WT mice during isoproterenol (ISO; 2 mg/kg) and caffeine (120 mg/kg) challenges. Further details are in the Online Data Supplement.

Results

Steady-State Binding of CaM to RyR2

Predepletion of Endogenous CaM by Suramin

RyR2s are concentrated at Z-lines, which is also true for CaM. Online Figure IAi shows the F-CaM Z-line striation pattern after 60 nmol/L F-CaM was washed in. Suramin (5 μmol/L) wash-in abolished striations within 60 s (Online Figure IAii), indicating F-CaM dissociation from Z-line sites. After suramin-dependent stripping and subsequent wash-out (20 minutes), F-CaM rebound at Z-lines, restoring striations (Online Figure IAiii). Before suramin treatment, F-CaM–binding kinetics was slow (time constant, \( \tau \approx 11.5 \) minutes; Online Figure IB), which represents F-CaM replacing endogenous RyR-bound CaM. After depleting endogenous RyR-bound CaM with suramin, F-CaM binding was much faster (\( \tau \approx 2 \) minutes; Online Figure IC), reflecting F-CaM binding to unoccupied binding sites. Binding maximum (Bmax) was not significantly altered by suramin pretreatment, indicating that suramin completely depleted endogenous Z-line–bound CaM and that CaM and suramin binding are completely reversible.

Steady-State CaM–RyR2 Binding Affinity

Strong FRET between F-FKBPs and F-CaM is effective in distinguishing CaM bound to RyR2 versus other CaM targets. After suramin pretreatment, 100 nmol/L F-FKBPs (donor) was washed in to saturate FKBP-binding sites on RyR2. Then, different F-CaM (acceptor) concentrations were washed in and allowed to reach steady state (20–120 minutes). RyR2-bound CaM is detected through FRET by sensitized emission. Figure IA shows fluorescent striations with F-FKBPs excitation at 488 nm and 2 different [F-CaM] in both donor (green) and acceptor (red) channels. In FRET-based Kd measurements, as [F-CaM] increases, donor fluorescence decreases (donor quenching), whereas acceptor fluorescence increases (enhanced acceptor fluorescence). The fluorescence intensity in the acceptor channel (difference between Z- and M-line fluorescence) is because of enhanced acceptor fluorescence (FRET) and reflects the amount of CaM specifically bound to RyR2. The binding isotherm in Figure IB is well described by a specific binding curve with a single binding site (Kd = 18 ± 2 nmol/L). We also calculated Kd from donor quenching (21 ± 3 nmol/L; Online Figure II) in agreement with that.

To test whether FRET occurs between 1 donor and 1 acceptor, in Figure IC we plotted donor signal (y axis) versus acceptor signal (x axis) for the same [F-CaM], from experiments shown in Figure IB. The dependence of donor fluorescence (F-FKBPs; decreasing because of FRET) on acceptor fluorescence (F-CaM; increasing FRET) is linear, consistent with bimolecular FRET. This suggests that FRET is exclusively

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<th>Nonstandard Abbreviations and Acronyms</th>
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<tr>
<td>Bmax binding maximum</td>
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<tr>
<td>CaM calmodulin</td>
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<td>CaMK Ca2+/CaM-dependent kinase</td>
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<td>CPVT catecholaminergic polymorphic ventricular tachycardia (VT)</td>
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<td>FKBP12.6 FK506-binding protein 12.6</td>
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<tr>
<td>FRAP fluorescence recovery after photobleaching</td>
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<td>FRET fluorescence resonance energy transfer</td>
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<td>HF heart failure</td>
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<tr>
<td>Kd dissociation constant</td>
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<td>Kd dissociation rate constant</td>
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<tr>
<td>Kd association rate constant</td>
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<tr>
<td>LTCC L-type Ca2+ channels</td>
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<tr>
<td>RyR2 ryanodine receptor type-2</td>
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<td>SR sarcoplasmic reticulum</td>
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between FKBP12.6 and CaM bound to the same face of the RyR2 (not to another nearby site).18

**Total CaM at Z-Line**

Using direct F-CaM excitation (543 nm) rather than FRET, we also measured the apparent $K_d$ for total CaM bound at the Z-line (ie, CaM bound to RyR2 plus other sites along the Z-line). Figure 1D shows Z-line striations at 2 different [F-CaM]. The plot of peak height reveals a single saturable binding component for F-CaM at the Z-line with $K_d=17\pm2$ nmol/L (Figure 1E). $K_d$ values for RyR2-bound CaM (Figure 1B) and for total CaM bound at the Z-line (Figure 1E) are similar. One possible explanation is that RyR2 is the quantitatively dominant CaM-binding site in the Z-line. The other explanation is that several CaM-binding sites have similar affinities. Through linear bath [F-CaM] calibration (Figure 1F), we can infer the concentration of Z-line–bound F-CaM in permeabilized myocytes. The maximum bound

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**Figure 1. Detection of calmodulin (CaM) binding to ryanodine receptor type-2 by fluorescence resonance energy transfer (FRET), and of total Z-line CaM binding by direct excitation.**

A. FRET images for 20 and 500 nmol/L acceptor [F-CaM], while keeping donor (F-FKBP) saturated. Donor fluorescence decreases and FRET signal increases at higher acceptor [F-CaM]. B, Acceptor (FRET) signal fit with single saturable binding isotherm (n=11–13). C, Donor quench and acceptor FRET are linearly related. D, Z-line images for direct F-CaM excitation at 20 and 500 [F-CaM]. E, Binding affinity fitted for CaM at Z-line (n=11–13), reflecting the $K_d$ and total CaM (binding maximum; $B_{\text{max}}$). F, Calibration of F-CaM fluorescence for different bath [F-CaM]. EAF indicates enhanced acceptor fluorescence; and FKBP, FKBP12.6.
CaM at the Z-lines (B_max; Figure 1E) is 1.2 μmol/L, similar to our previous results and consistent with the B_max for FKBP12.6 in rat myocytes.

**Kinetics of CaM Binding to RyR2**

**FRET-Based Measurement of k_on and k_off During Wash-In and Wash-Out in Myocytes**

After CaM depletion, permeabilized myocytes were saturated with F-FKBP (100 nmol/L). Using FRET, we characterized CaM–RyR2 association (k_on) and dissociation (k_off) rate constants in F-CaM wash-in/out experiments (Figure 2A). During wash-out, fluorescent striations gradually dissipated along a single exponential decay (τ=4.5 minutes), which corresponds to k_off=0.22±0.01/min (n=5). For wash-in, CaM–RyR2 association rate is a function of k_on, k_off, and [F-CaM] (k_wash-in=k_on[CaM]+k_off). Using the k_off and the wash-in data, we calculated k_on=18.9±1.6×10^6/min per mol/L (n=5). These k_on and k_off values correspond to K_d=12±1 nmol/L (k_off/k_on), which agrees with the above steady-state binding results.

**Fluorescence Recovery After Photobleaching–Based Measurement of k_on and k_off in Myocytes**

Permeabilized myocytes were first equilibrated with saturating F-FKBP (100 nmol/L) and with [F-CaM] in the K_d range. After photobleaching the acceptor by 543 nm excitation in the central region of the cell (Figure 2B), we monitored striation recovery (fluorescence recovery after photobleaching, FRAP). FRAP reflects recovery of CaM–RyR2 binding, in which the photobleached RyR-bound F-CaM dissociates and is replaced by fresh F-CaM from the bath. In Figure 2C, k_FRAP was fit to a single exponential function for 2 different bath [F-CaM], on which FRAP is dependent (k_FRAP=k_on[F-CaM]+k_off). The values of k_on and k_off are obtained from linear regressions (Figure 2D). Both k_on=14.5±2.5×10^9/min per mol/L and k_off=0.144±0.05/min agree well with the wash-in/out experiments (Figure 2A) and give a similar K_d (10±2 nmol/L).

**Percentage of Total Z-Line CaM That Is RyR2-Bound**

KI mice with genetically disrupted CaM–RyR2 interaction are used here (along with WT mice) to estimate the percentage of total Z-line–bound CaM that is RyR2-bound. These KI mice express RyR2 with a triple mutation (RyR2–W3587A/L3591D/F3603A, RyR2ADA/+), which prevents high-affinity binding of CaM. Homozygous (RyR2 ADA/ADA) mice all die within 16 days of birth with a severe HF phenotype, loss of T-tubule/junctional couplings, and ≈70% decrease in RyR2 expression, making them unsuitable here. However, the heterozygous mice (RyR2 ADA+/+) have no evidence of HF or...
structural defects and exhibit normal lifespan and RyR2 expression levels.\(^{11}\) We find that RyR\(^{\text{ADA/+}}\) myocytes exhibit reduced F-CaM binding both to the RyR2 (by FRET) and at the Z-line (by direct F-CaM excitation; Figure 3A and 3B). For RyR2\(^{\text{ADA/+}}\), there is a 50\(\pm\)2\% reduction in FRET at saturating [F-CaM] (FRET\(_{\text{max}}\)) with no significant change in K\(_d\) (Figure 3C). This fits the expectation that in RyR2\(^{\text{ADA/+}}\) mice \(\approx\)50\% of the RyR2 monomers have the triple mutation and defective CaM binding. There is also a 46\(\pm\)2\% reduction of total CaM at the Z-line (Figure 3D), which is primarily because of the 50\% decrease in CaM binding to RyR2. This suggests that \(\approx\)92\% of Z-line–localized CaM is bound to RyR2.

We also measured RyR2 monomer expression as total binding (B\(_{\text{max}}\)) of FKBP12.6 (Online Figure III).\(^{22}\) FKBP12.6 binding was identical in RyR2 ADA/+ versus WT myocytes, confirming an unaltered total number of RyR2 monomers, in line with unaltered B\(_{\text{max}}\) of \([\text{H}]\)-ryanodine binding in these mice.\(^{11}\)

**Ca\(^{2+}\) Sparks in Permeabilized RyR2\(^{\text{ADA/+}}\) Ki Mouse Myocytes**

Saponin-permeabilized myocytes are powerful tools for the evaluation of diastolic SR Ca\(^{2+}\) leak. The free [Ca\(_i\)], can be tightly controlled to avoid complications because of LTCCs, which can trigger RyR2-mediated Ca\(^{2+}\) release. However, a potential disadvantage of this method is that some cellular contents (such as endogenous proteins) can be lost because of wash-off. Because the endogenous CaM associated with RyR2 may be washed off within 10 minutes (Figure 2A), we modified the permeabilization protocol for Ca\(^{2+}\) spark measurements to limit this disadvantage. For Ca\(^{2+}\) spark measurements, myocytes were exposed to saponin (50 \(\mu\)g/mL) for only 20 to 30 s instead of 3 minutes as used above for CaM wash-in. Online Figure IV shows that after 20 to 30 s of permeabilization Ca\(^{2+}\) sparks were readily detected in myocytes after 20-minute exposure to F-FKBP, F-CaM, and fluo-4. However, there was no visible striated pattern observable for F-FKBP, F-CaM, or FRET. This indicates that fluo-4 can enter the cell with this gentler permeabilization, but proteins (eg, CaM and FKBP12.6) cannot. With the 3-minute permeabilization protocol, both FRET striation pattern and Ca\(^{2+}\) sparks were detected, indicating that all 3 probes entered the cell. This shows that 20- to 30-s permeabilization can also effectively limit FKBP12.6 and CaM wash-out during the time of Ca\(^{2+}\) spark measurement. Thus, we can undertake Ca\(^{2+}\) spark measurement in the relatively native cellular environment with respect to CaM and FKBP12.6 concentrations.

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**Figure 3.** Percentage of Z-line calmodulin (CaM) that is on ryanodine receptor type-2 (RyR2). A, Fluorescence resonance energy transfer (FRET) images in saturating F-CaM and F-FKBP12.6 conditions for wild type (WT) and RyR2\(^{\text{ADA/+}}\). B, Z-line–bound F-CaM images (direct F-CaM excitation) for WT and RyR2\(^{\text{ADA/+}}\). C, Steady-state CaM binding, with binding maximum (B\(_{\text{max}}\)) normalized to WT level and with K\(_d\) inferred (n=12–20). D, Total Z-line associated F-CaM with B\(_{\text{max}}\) and K\(_d\) for RyR2\(^{\text{ADA/+}}\) and WT mice (n=14–20). EAF indicates enhanced acceptor fluorescence; and FKBP12.6, FK506-binding protein 12.6.
Using this approach, we measured diastolic SR Ca\textsuperscript{2+} leak as Ca\textsuperscript{2+} sparks within 15 to 20 minutes of permeabilization. Figure 4A and 4B shows that, at 50 nmol/L [Ca\textsuperscript{2+}], spark frequency was significantly higher in RyR2\textsuperscript{ADA+} versus WT mice (8.9±0.3 versus 6.4±0.5/s per 100 μm), which tended to lower SR Ca\textsuperscript{2+} content in RyR2\textsuperscript{ADA+} versus WT (albeit insignificantly; Figure 4B). Ca\textsuperscript{2+} spark frequency depends steeply on SR Ca\textsuperscript{2+} content,\textsuperscript{37} such that the new steady-state spark frequency underestimates the magnitude of the primary effect. The increased Ca\textsuperscript{2+} spark frequency could not be prevented by the specific CaMKII inhibitor autocamtide-2–related inhibitory peptide, AIP (Figure 4B), ruling out a contribution of CaMKII. These results are consistent with half of the RyR2 monomers lacking CaM, allowing higher RyR2 activity.

In a second series of spark experiments, we compared the effect of endogenous CaM (as above) with CaM wash-out and readmission in WT and RyR2\textsuperscript{ADA+} mice (Online Figure V). For WT, CaM wash-off increased Ca\textsuperscript{2+} spark frequency, Ca\textsuperscript{2+} spark full duration at half-maximum, and Ca\textsuperscript{2+} full width at half-maximum. These effects were reversed with CaM re-addition. However, in RyR2\textsuperscript{ADA+} myocytes, the baseline Ca\textsuperscript{2+} spark frequency, Ca\textsuperscript{2+} full duration at half-maximum, and Ca\textsuperscript{2+} full width at half-maximum were already high (comparable to that in CaM-free WT myocytes) and were not influenced by CaM wash-out or readmission. Thus, even 500 nmol/L CaM cannot reverse the high Ca\textsuperscript{2+} spark frequency caused by defective CaM–RyR2 interaction (Online Figure V).

We also increased intracellular [Ca\textsuperscript{2+}] to 100 nmol/L, close to the threshold for Ca\textsuperscript{2+} waves in WT mouse myocytes, to further test the propensity for delayed afterdepolarizations or arrhythmogenesis. The increased [Ca\textsuperscript{2+}] mimics a Ca\textsuperscript{2+} loading stress in permeabilized cells, because both cytosolic and SR Ca\textsuperscript{2+} load are increased. In RyR2\textsuperscript{ADA+} mice, ≈90% of myocytes produced Ca\textsuperscript{2+} waves versus only ≈10% of myocytes for WT mice (Figure 4C and 4D), again without a significant difference in Ca\textsuperscript{2+} SR load (Figure 4D). The caffeine-induced Ca transients were induced during sweeps where no waves occurred, to limit underestimation of SR Ca\textsuperscript{2+} content that would occur in the wake of a wave. All these data indicate that the ADA CaM-binding mutation in RyR2 increases diastolic SR Ca\textsuperscript{2+} leak under resting conditions and increases propensity for arrhythmogenic Ca\textsuperscript{2+} wave production under moderate Ca\textsuperscript{2+} loading conditions.

**Ca\textsuperscript{2+} Transients in RyR2\textsuperscript{ADA+} KI Mouse in Intact Myocytes**

We also measured Ca\textsuperscript{2+} transients in intact ventricular myocytes (Figure 5A) with or without ISO (50 nmol/L), and SR Ca\textsuperscript{2+} content was evaluated through rapid caffeine application. In 1 Hz field stimulation at baseline, RyR2\textsuperscript{ADA+} myocytes behave similar to WT in Ca\textsuperscript{2+} transient amplitude, time constant of Ca\textsuperscript{2+} transient decline, SR Ca\textsuperscript{2+} content, and fractional SR Ca\textsuperscript{2+} release (Figure 5B–5E). Although exposure to ISO (50 nmol/L) had similar effects on Ca\textsuperscript{2+} transient amplitude in both WT and RyR2\textsuperscript{ADA+}, the time constant of [Ca\textsuperscript{2+}]\textsuperscript{3} decline was longer, SR Ca\textsuperscript{2+} content was lower, and fractional SR Ca\textsuperscript{2+} release was increased in RyR2\textsuperscript{ADA+} versus WT (Figure 5C–5E). The more sensitive RyR2\textsuperscript{ADA+} and higher Ca\textsuperscript{2+} loading in 50 nmol/L ISO may cause the prolonged twitch [Ca\textsuperscript{2+}]\textsuperscript{3} decline (delayed shut-off of release, as demonstrated under analogous conditions in CaMKII\textsubscript{δ}-overexpressing mice that lack phospholamban).\textsuperscript{38} The exacerbated leak in RyR2\textsuperscript{ADA+} mice may also limit the rise in SR Ca\textsuperscript{2+} content induced by ISO, such as the reduced maximal achievable SR Ca\textsuperscript{2+} content in HF or ISO-treated myocytes.\textsuperscript{24,39} Figure 6 also shows that, in RyR2\textsuperscript{ADA+} myocytes, ISO increased the propensity for Ca\textsuperscript{2+} waves that trigger action potentials. That is, the transition from Ca\textsuperscript{2+} wave to spatially synchronized global Ca\textsuperscript{2+} release was necessarily synchronized by a triggered action potential.
These triggered events could reflect increased arrhythmogenic risk in RyR2^ADA/+^ mice, analogous to CPVT.

**Ventricular Arrhythmia in RyR2^ADA/+^ KI Mouse**

To test whether the RyR2^ADA/+^ KI mice are more susceptible to CPVT-like arrhythmias, we measured ECGs in intact mice during injection of ISO plus caffeine as has been performed in CPVT mouse models. Figure 7A shows that RyR2^ADA/+^ KI mice developed severe stress-inducible ventricular arrhythmia. All RyR2^ADA/+^ mice exhibited sustained bigeminy (>20 minutes), a precursor for ventricular tachycardia (VT; Figure 7B). In 60% of RyR2^ADA/+^ mice, bidirectional VT was induced (Figure 7B), and episodes of VT were frequent (4.5 per mouse) in RyR2^ADA/+^ mice. In contrast, none of the WT mice exhibited inducible arrhythmia in this protocol.

**Binding Affinity of CaM/FKBP12.6 to RyR2 in HF Myocytes**

We measured CaM–RyR2 affinity in a rat HF model induced by coronary ligation. Twelve weeks postinfarct, these rats exhibited increased heart/body weight ratio, increased left ventricular diastolic dimensions, and decreased fractional shortening. Other HF molecular markers (ANP, BNP, TNF-α) are also increased in this model. The HF rats used here had reduced fractional shortening (<20% versus normal; 46%) and elevated left ventricular diastolic dimensions (Online Figure VI). We used FRET to measure the CaM–RyR2 affinity. In HF versus normal myocytes, the CaM–RyR2 affinity was ≈3-fold lower (K_d = 51±4 nmol/L; Figure 8A). We also performed paired experiments with sham rats, with [F-CaM] near the K_d (FRET max; Figure 8B). At [F-CaM] near WT K_d, changes in affinity are readily detected, and binding was significantly lower in HF. FRET_max was unaltered.

FKBP12.6 was suggested to also be a critical RyR2 stabilizer, with decreased affinity in HF (although this is controversial). Using methods previously described, we measured, for the first time within HF myocytes, the FKBP12.6–RyR2 affinity (K_d = 0.8±0.1 nmol/L; Figure 8C). This is almost identical to the K_d we previously measured in normal rat myocytes under the same conditions (0.7±0.1 nmol/L). We also performed paired experiments (as for CaM). We found no significant changes in FKBP12.6–RyR2 binding either at subsaturating or saturating [F-FKBP] in sham versus HF rat myocytes (Figure 8D).
Discussion

Here we resolved CaM binding to RyR2 versus other Z-line–binding partners and characterized CaM–RyR2 binding properties (Kd and on/off rates) in permeabilized ventricular myocytes by 3 independent methods (Online Table I). RyR2ADA/+ KI mice with disrupted CaM–RyR2 binding were used to determine that >90% of Z-line CaM is bound to RyR2. Functional consequences of reduced CaM binding to RyR2 were detected using Ca2+ measurements in WT versus RyR2 ADA/+ KI myocytes, with CaM wash-out from WT myocytes, and arrhythmogenesis tests in mice. We also found CaM–RyR binding affinity to be reduced in HF myocytes. These results provide important new insights into understanding CaM–RyR interactions in normal and HF myocytes.

CaM–RyR2 Binding Properties in Myocytes

CaM, as a key regulator of RyR2 function, may be important in myocyte Ca2+ homeostasis in HF, cardiac hypertrophy, and CPVT. But in situ CaM–RyR2 binding properties were previously unknown. Using 3 independent methods, we demonstrated that CaM binds to RyR2 with high affinity (Kd = 10–20 nmol/L) in myocytes at 50 nmol/L [Ca2+]i. Considering the measured free [CaM] in myocytes (50–75 nmol/L), the majority of RyR2 monomers (~70%–90%) are CaM-bound, such that CaM can influence local [Ca2+]i by inhibiting RyR2 opening at physiological [Ca2+]i.12,13,23,26,42 The CaM–RyR2 binding affinity we measured in myocytes is higher than in cell lysates and SR vesicles.17,27 This could be because of differences in experimental conditions or subcellular fractionation effects. In SR vesicles, essential partners from the cellular environment may be lost, or post-translational modifications of RyR2 might occur (eg, phosphorylation or oxidation), which can alter CaM–RyR2 affinity.23,43 Using 3 methods, we measured similar CaM–RyR2 affinities that are consistent with CaM’s physiological function.13,23 This supports that our Kd measurements (10–20 nmol/L) are valid for the CaM–RyR2 binding affinity in the native cardiac myocyte environment.

According to our data, CaM–RyR2 has a relatively slow koff (~0.2/min), meaning that the average dwell time for CaM on RyR2 is ~5 minutes under resting conditions. In addition, high [Ca2+]i (500 nmol/L) strengthens CaM binding to RyR/Z-lines and greatly slows CaM dissociation.13,24 Hence, increased CaM–RyR2 affinity during the Ca2+ transient (<1 s) would further enhance CaM saturation at all RyR2 monomers in the physiological beat-to-beat situation. Furthermore, it was proposed that CaM can switch between 2 binding sites separated by ~33Å on RyR1 during each cardiac cycle.44,45 The 2-site switch involves 2 sets of association and dissociation process, which seems unlikely to occur within the short systolic time, according to our measured kinetics. Taken together, these suggest that CaM is a resident RyR2-associated protein anchored at least to residues 3583 to 3603. This interpretation is consistent with a recent cryo-EM report placing apo- and Ca-CaM at the same location within the RyR2 map.46 However, we cannot exclude the possibility of beat-to-beat RyR2 regulation by CaM because homozygous RyR2ADA/ADA myocytes exhibit prolonged Ca2+ transient and spark durations. That is consistent with the possible importance of resident CaM for RyR2 shut-off.23,24,47 During a Ca2+ transient, RyR2 opening and conformation change could alter anchored CaM function, where binding of Ca2+ to CaM would facilitate the termination of Ca2+ release.

Nearly All Z-Line–Bound CaM Is on RyR2

An important finding here is that >90% of the total Z-line–associated CaM is bound to RyR2, that is, ~1.2 μmol/L (Figure 1E). This is consistent with our FKBP12.6 Bmax.
measurements\textsuperscript{22} and with the estimated concentration of RyR2 monomers in rat ventricular myocytes,\textsuperscript{48} implying that there may be \(\approx 70\) nmol/L of non-RyR2 CaM-binding sites at the Z-line. Because the LTCC also binds CaM at the Z-line, and there are \(\approx 32\) RyR2 monomers per LTCC in rat myocytes,\textsuperscript{48} LTCC would bind \(\approx 40\) nmol/L CaM, consistent with our

\[\text{CaM} \text{-- RyR2 Binding in Cardiomyocytes}\]

**Figure 7.** Effect of caffeine plus isoproterenol (ISO) on ventricular arrhythmia (RyR2\textsuperscript{ADA/+}).

A, Representative ECG recording for RyR2\textsuperscript{ADA/+} mice (ADA/+); severe ventricular arrhythmia (sustained bigeminy, bidirectional ventricular tachycardia [VT] and polymorphic VT) were induced by ISO plus caffeine. B, 100% of RyR2\textsuperscript{ADA/+} mice exhibit sustained bigeminy (> 20 min); 60% RyR2\textsuperscript{ADA/+} mice exhibit typical bidirectional VT, and VT (bidirectional or polymorphic) episode is 4.5 per mouse.

**Figure 8.** Calmodulin (CaM) and FK506-binding protein 12.6 (FKBP12.6) binding to ryanodine receptor type-2 (RyR2) in heart failure (HF) myocytes. A, Fluorescence resonance energy transfer (FRET) detection and steady-state concentration–dependant binding; \(K_d\) of CaM–RyR2 was measured in HF myocytes (n=18). B, \(K_d\) for FKBP12.6–RyR2 was measured by steady-state binding in HF myocytes (n=18–22). C, For \(K_d\) range (20 nmol/L), there is a significant decrease in CaM–RyR2 associate rate for HF, but with same binding maximum (\(B_{\text{max}}\), in saturating condition). D, For \(K_d\) range or saturating condition (\(B_{\text{max}}\)), the FKBP12.6–RyR2 associate rate is unchanged for control and HF myocytes. EAF indicates enhanced acceptor fluorescence.
findings. It is clear that RyR2 is the quantitatively dominant CaM-binding site at the Z-line. Total cellular [CaM] in rat cardiac myocytes is 2 to 6 μmol/L and varies somewhat with species.49 We infer that a reduction in CaM–RyR2 binding affinity, as in HF or CPVT,25–27 might also shift CaM’s distribution among different binding targets. Because free [CaM] is only 50 to 100 nmol/L in myocytes (>95% is bound) even at diastolic [Ca2+]i, there is likely to be competition among target sites for available CaM.30

Defective CaM–RyR2 Interaction and SR Ca2+ Release, CPVT
Here, RyR2ADA/+ mice13 were used to assess CaM binding at the RyR2 and Z-line quantitatively, but this also alters SR Ca2+ leak, which provides a functional correlate. Homozygous RyR2ADA/ADA mice have altered SR Ca2+ release, but also exhibit profound HF, death within ≈2 weeks of birth, 70% reduction in RyR2 expression, and T-tubule disorganization.13,24 Remarkably, RyR2ADA/+ mice have normal lifespan, myocyte ultrastructure, and RyR2 expression with no hypertrophy.13 This is similar to KI mice carrying a CPVT1 mutation (RyR2-R2474S),25,26 where heterozygous mice survive without hypertrophy similar to WT, but the homozygous KI is lethal. Decreased CaM–RyR2 binding affinity has also been reported in CPVT1 KI mice under stress conditions known to be arrhythmogenic.27 Thus, the RyR2ADA/+ mice studied here resemble a CPVT1 model, and we also assessed abnormal SR Ca2+ release and stress-induced arrhythmic events in these mice.

Baseline Ca2+ transients were similar between RyR2ADA/+ and WT mice, consistent with previous hemodynamic data, indicating functional normalcy of these mice (versus WT littermates).13 RyR2ADA/+ myocytes exhibited a moderate increase in Ca2+ spark frequency at baseline. The moderate Ca2+ leakiness may explain why the heterozygous mice survive as long as WT controls (unlike homozygous RyR2ADA/ADA). Recently, Yamaguchi et al13 showed that the single RyR2-L3591D mutant (which also reduces RyR2–CaM binding), when homozygous in KI mice, increases RyR2 open probability at diastolic [Ca2+]i, but is much better tolerated versus the RyR2ADA/ADA triple mutation. These results are consistent with the CPVT phenotype, where the CPVT patients are completely normal under resting condition.

Catecholamine challenge (ISO) in RyR2ADA/+ further exacerbated SR Ca2+ leak, limited the ISO-induced lusitropy and SR Ca2+ loading, but the Ca transients were still similarly enhanced (versus WT; Figure 5). We infer that this is because there is increased fractional SR Ca2+ release in the RyR2ADA/+ mice with ISO. So this mouse seems adapted to have normal adrenergic inotropic response, but the SR is pushed closer to instability. In particular, we suspect that the limited lusitropic effect and reduced SR Ca2+ content after ISO in RyR2ADA/+ mice may be because of the dramatically increased SR Ca2+ leak, especially because Yamaguchi et al13 found that SR Ca2+-ATPase–dependent uptake rate in RyR2ADA/+ mice was normal. This agrees with single-channel RyR2 recordings suggesting that defective CaM–RyR2 interaction delays RyR2 closure.23

Despite the relative normalcy of Ca2+ transients in RyR2ADA/+ myocytes, the exacerbation of leak may limit maximal SR Ca2+ content, just as seen for both ISO treatment alone and HF alone.25,39 This might limit cardiac reserve. More importantly, this enhanced leak in RyR2ADA/+ increases Ca2+ waves and triggers activity in myocytes and whole animal arrhythmias in response to catecholamine challenge (Figures 4, 6 and 7). This again recapitulates the situation in CPVT1 KI mice and patients.25,29 In particular, the bidirectional VT seen in RyR2ADA/+ (but not WT mice) is clinically diagnostic for CPVT.29 Although this RyR2 ADA mutation is not a known disease-linked mutation in humans, it causes a CPVT-like phenotype. There are also 2 recent CaM mutations that are associated with CPVT in patients,30 and the CPVT phenotype may well result from effects of these mutant CaMs analogous to our RyR2ADA/+ mouse results here.

We cannot exclude the possibility that the ADA triple mutation alters RyR2 domain interactions, which might also contribute to the functional effects observed. However, the similarity between the RyR2ADA/+ and simply depleting CaM from the WT RyR2 (Online Figure V) convinces us that the loss of CaM binding is the major factor in RyR2ADA/+ mice causing abnormal SR Ca2+ leak and arrhythmias. We recently showed that CaM binding can stabilize RyR2 conformation in a more stably closed (zipped) state where access of an unzipping peptide is supressed.52

CaM–RyR2 Binding Affinity in HF Myocytes
Previous work in HF rabbit and canine models demonstrated reduced RyR2-bound CaM, based on immunoprecipitation and biochemical analysis,25,26 but total CaM expression was unaltered.31 Here, we directly measured CaM and FKBP12.6 affinity for RyR2 in situ in HF myocytes. We found a 3-fold lower CaM affinity in HF myocytes, whereas FKBP12.6 affinity was unaltered. This quantitative information allows novel inferences. Assuming that free [CaM] in HF myocytes is 50 to 100 nmol/L,34 the saturation of RyR2 monomers with CaM would be expected to drop from 70%–90% to 50%–70% in HF. This lower RyR2 saturation with CaM may have an analogous functional effect to those that we found in the RyR2ADA/+ mice, where only 50% of RyR2 can bind CaM. The implication is that reduced CaM binding to RyR2 may contribute to the known increased SR Ca2+ leak in HF25 and to the incidence of arrhythmias, which are responsible for 50% of HF deaths.1 Furthermore, attenuated CaM–RyR2 association in HF could allow other intracellular CaM targets of lower affinity to be better activated, because RyR2 is a major CaM site in myocytes.31 One such site is CaMKII, which is activated in HF and contributes to SR Ca2+ leak and arrhythmogenesis by phosphorylation and activation of RyR2.28 Thus, decreased CaM–RyR2 association in HF may synergize with other regulatory pathways to exacerbate cardiac dysfunction and arrhythmias. Overall, the HF-associated Sr Ca2+ leak is a complicated process involving several factors (eg, CaM, CaMKII, FKBP12.6, redox modification). Here, for the first time, we measured directly the in situ binding affinity of RyR2 for FKBP12.6 in HF myocytes, but found it to be unaltered versus control. We conclude that CaM binding to RyR2 may be an important physiological regulator of RyR2 gating in cardiac myocytes and that defects in this binding in HF or CPVT might constitute an important molecular mechanism of triggered arrhythmias suitable for therapeutic targeting.
Disclosures

None.

References


**What Is Known?**

- Calmodulin (CaM) binds to multiple cellular targets, including the sarcoplasmic reticulum (SR) Ca2+ release channel (ryanodine receptor type-2, RyR2) in cardiomyocytes. CaM binding reduces RyR channel opening and can reduce SR Ca2+ leakiness.

- Abnormal SR Ca2+ leak is thought to contribute to dysfunction and arrhythmias in catecholaminergic polymorphic ventricular tachycardia (CPVT) and heart failure (HF).

- FK506-binding protein 12.6 (FKBP12.6) binds to RyR2 with high affinity and may also stabilize RyR gating, but results are controversial.

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

- In the native cardiac myocyte environment with 50 nmol/L [Ca2+]i, CaM binds to RyR2 (and at the Z-line) with high affinity (Kd ≈ 15 nmol/L).

- More than 90% of CaM at the Z-line is RyR2-bound in cardiac myocytes.

- CaM dissociation from RyR2 or lack of binding to mutant RyR2 can lead to abnormal SR Ca2+ release in the form of increased Ca sparks, Ca waves, triggered activity, and arrhythmias at the whole animal level.

- These arrhythmogenic features recapitulate RyR2 changes seen in CPVT and HF.

**Novelty and Significance**

- In a post–myocardial infarction (MI) rat HF model, RyR2–CaM binding affinity is reduced =3-fold, whereas FKBP12.6 affinity is unaltered.

CaM is known as a mediator of Ca2+ signals, and it can bind to and alter RyR properties. However, little quantitative data are available about CaM–RyR interaction and function in native cardiac myocytes. We used confocal fluorescence microscopy to measure the in situ binding kinetics and affinity of CaM for RyR in normal myocytes and in myocytes from mice expressing a mutant RyR that cannot bind CaM (CPVT-like model) and from rats with post-MI–induced HF. Using fluorescence resonance energy transfer, we can distinguish RyR2-bound CaM from CaM bound to other sites in the myocyte. More than 90% of CaM that is already highly concentrated along the Z-lines of the myocyte is bound directly to RyR. We also found that CaM binding to RyR was reduced in the CPVT-like and HF models and that it causes enhanced SR Ca2+ leak leading to arrhythmogenic events. Reduced CaM binding to RyR in cardiac myocytes resulting in enhanced SR Ca2+ leak may be an important contributor to both reduced systolic function and arrhythmias.
Cardiac Myocyte Z-line Calmodulin is mainly RyR2-bound and reduction is arrhythmogenic and occurs in Heart Failure
Ya Cuo Oda Chakraborty Chen Uchino U Min Knowlton Fruen Cornea Meissner and Bers

ONLINE METHODS
Cardiac Myocyte Isolation and Heart Failure Model
Ventricular myocyte isolation was carried out as previous described. Ventricles were dispersed, filtered, and rinsed to obtain myocytes which were placed in normal Tyrode’s (NT) solution (in mM): NaCl 140, KCl 4, MgCl2 1, CaCl2 2, glucose 10, HEPES 5, pH 7.4. Experiments were done at room temperature (23°C).

We used a rat coronary ligation model of HF using Sprague-Dawley rats, as previously described. This protocol produces HF 12 weeks after surgery. All animal procedures were approved by the University of California, Davis Animal Research Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Synthesis of Fluorescent FKBP12.6/CaM Probes
Single-cysteine variants of FKBP12.6 and CaM were labeled with Alexa Fluor 488 (AF488) and Alexa Fluor 568 (AF568) maleimides, as previously described. Accordingly, we labeled the FKBP12.6 mutant at position 14 with green fluorescent AF488 as FRET donor (denoted F-FKBP). We labeled T34C-CaM with red fluorescent AF568 as FRET acceptor (denoted F-CaM). These F-FKBP and F-CaM bind to and regulate RyRs in SR vesicles like wild type proteins. The Alexa Fluor dyes were purchased from Invitrogen.

Cell Permeabilization
Myocytes in relaxation solution were permeabilized by saponin (50 μg/ml). For experiments with fluorescent proteins vs. Ca2+ indicator, myocytes were permeabilized for 3 min vs. 20-30 s, respectively. Relaxation and permeabilization solution contain (mM): EGTA 0.1, HEPES 10, K-aspartate 120, free MgCl2 1, ATP 5, reduced glutathione 10, phosphocreatine di-Tris 5 with or without saponin at pH 7.4. Internal solution after permeabilization was similar, but [EGTA] was 0.5 mM, free [Ca2+] was 50 nM (calculated using MaxChelator), creatine phosphokinase was 5 U/ml; phosphocreatine was 10 mM; dextran (MW: 40,000) was 4% at pH 7.2.

FRET in Permeabilized Ventricular Myocytes
After permeabilization, myocytes were exposed to internal solution. F-FKBP and F-CaM were added to the bath as previously described, at concentrations as indicated. F-FKBP (donor) was excited at 488 nm, and emission was recorded through a 515±15 nm band pass filter. For FRET, F-CaM fluorescence emission was detected using a 600 nm long pass filter. F-CaM fluorescence was also directly excited at 543 nm, and detected as above.

Fluorescence Recovery after Photobleach (FRAP)
Permeabilized myocytes were incubated with the FRET pair: 100nM F-FKBP (donor) and a range of [F-CaM]. F-CaM bound on RyR2 specifically was detected through FRET by sensitized emission. At steady state, F-CaM was photobleached in a region of the cell using 543 nm excitation at high laser power (90%) for 50s, resulting in >95% F-CaM fluorophore destruction in that region. Thereafter the patterned striation recovery of the photobleached region was recorded via FRET (488 nm excitation, >600 nm detection). This reflects recovery of RyR2 bound CaM through reversible binding, which was modeled as a first order reaction. The observed recovery rate constant kFRAP was fitted to a single-exponential function, and is an integrated measure that depends on both kon and koff (kFRAP= kon[F-CaM] + koff). Since kFRAP is [F-CaM] dependent, we measured kFRAP at various [F-CaM] near the Kd value (after depletion of endogenous CaM) to determine kon (slope) and koff (intercept) in via linear regression (F-CaM diffusion is much faster than FRAP and not rate-limiting).

Confocal Ca2+ Imaging
Ca2+ sparks were recorded in saponin-permeabilized ventricular cardiomyocytes, which were superfused by internal solution with free 50 nM/100nM [Ca2+] and 25 μM Fluo-4. CaMKII activity was inhibited by AIP (1 μM). Intact ventricular myocytes were loaded with Fluo-4 AM (5 μM, Molecular Probes) and Ca2+ transients were recorded as previously described.

ECC Recording
ECGs were recorded in limblead I and III configuration. RyR2ADΔK+ KI and WT mice were anesthetized with 5% isoflurane on a heating pad at 30ºC. Then Is oflurane was reduced to 0.6% and administered by intraperitoneal (i.p.) injection and ECG was then recorded for 30-50 min.

Statistics
Data are means +/-SEM, with significance assessed using paired or unpaired Student’s t test, as appropriate.

References
Online Fig I. Suramin can predeplete endogenous CaM. A, confocal images of saponin-permeabilized myocytes incubated with 60 nM F-CaM: (i), after 40 min incubation when fluorescent striation reached $B_{\text{max}}$. (ii), 5 µM suramin totally abolishes fluorescent striations in 1 min. (iii), after suramin washoff (20 min) and reintroduction of F-CaM, fluorescent striation reaches $B_{\text{max}}$ much faster. B, time course for 60 nM F-CaM wash-in, immediately after permeabilization. C, time course for 60 nM F-CaM wash-in, after treatment of suramin.

Online Fig II. $K_d$ fitting from donor quench with increasing [F-CaM]

$$F_{(\text{donor})} = C - \{B_{\text{min}} \times [\text{CaM}] / (K_d + [\text{CaM}])\}$$

$K_d = 21 \pm 3 \text{ nM}$
Online Fig III. Total binding ($B_{\text{max}}$) of FKBP12.6 for WT and RyR2$^{ADA/+}$. A, Confocal images of F-FKBP-12.6 binding for WT and RyR2$^{ADA/+}$ mice. B, $B_{\text{max}}$ for FKBP12.6 is unaltered in WT vs RyR2$^{ADA/+}$ mice.

Online Fig IV. Effect of saponin permeabilization time. A, 30 sec permeabilization (50 µg/ml saponin) only allows fluo-4 dye entry. B, 3 min permeabilization allows F-FKBP12.6, F-CaM and fluo-4 entry.
Online Fig V. Ca²⁺ spark frequency and characteristics in permeabilized myocytes. A, Ca²⁺ spark frequency. B, Ca²⁺ spark full duration at half-maximum (FDHM). C, Ca²⁺ spark amplitude. D, Ca²⁺ spark full width at half-maximum (FWHM).
Online Fig VI. Altered cardiac function in HF rats at 12 weeks post-infarct. Left ventricular diastolic dimension (LVDd), Left ventricular end systolic dimension (LVDs) and Fractional shortening measured by echocardiography in Sham and HF (5 rats/group). 

Online Fig VII. The effect of FKBP12.6 and CaM to each other binding.
Online Fig VIII. Representative ECG recording for WT mice with ISO plus caffeine treatment.

Online table 1. In situ CaM-RyR2 interaction properties

<table>
<thead>
<tr>
<th>Condition</th>
<th>$k_{on}$ (x10^6min^-1M^-1)</th>
<th>$k_{off}$ (min^-1)</th>
<th>$K_d$ (nM)</th>
</tr>
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<td>Wash-in/off</td>
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<td>12 ± 0.9</td>
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<td>FRAP</td>
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<td>10 ± 2</td>
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<tr>
<td>Steady-state</td>
<td>--</td>
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<td>18.2 ± 2</td>
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