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Free and Bound Intracellular Calmodulin Measurements in Cardiac Myocytes

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Abstract

Calmodulin (CaM) is a ubiquitous Ca^{2+} binding protein and Ca^{2+} -CaM activates many cellular targets and functions. While much of CaM is thought to be protein bound, quantitative data in cardiac myocytes is lacking regarding CaM location, $[\text{CaM}]_{\text{free}}$ and CaM redistribution during changes in $[\text{Ca}^{2+}]_i$. Here, we demonstrated that in adult rabbit cardiac myocytes, CaM is highly concentrated at Z-lines (confirmed by Di-8-ANEPPS staining of transverse tubules) using three different approaches: immunocytochemistry (endogenous CaM), Alexa Fluor 488 conjugate CaM (F-CaM) in both permeabilized cells (exogenous CaM) and in patch clamped intact cells (via pipette dialysis). Using 100 nM $[\text{CaM}]_{\text{free}}$ we washed F-CaM into permeabilized myocytes and saw a two-phase (fast and slow) CaM binding curve with a plateau after 40 min of F-CaM wash-in. We also measured myocyte $[\text{CaM}]_{\text{free}}$ using two modified null-point titration methods, finding $[\text{CaM}]_{\text{free}}$ to be 50–75 nM (which is only 1% of total $[\text{CaM}]$). Higher $[\text{Ca}^{2+}]_i$ increased CaM binding especially on nucleus and Z-lines and significantly slowed F-CaM dissociation rate when F-CaM was washed out of permeabilized myocytes. Additionally, in both permeabilized and intact myocytes, CaM moved into the nucleus when $[\text{Ca}^{2+}]_i$ was elevated, and this was reversible. We conclude that $[\text{CaM}]_{\text{free}}$ is very low in myocytes even at resting $[\text{Ca}^{2+}]_i$, indicating intense competition of CaM targets for free CaM. Bound CaM is relatively concentrated at Z-lines at rest but translocates significantly to the nucleus upon elevation of $[\text{Ca}^{2+}]_i$, which may influence activation of different targets and cellular functions.

Keywords

CaM; $[\text{CaM}]_{\text{free}}$; CaM binding; CaM redistribution

Introduction

Calmodulin (CaM) is highly conserved among different species (148 amino acids, molecular weight ~16.7 kDa). CaM exists as ApoCaM (Ca^{2+} free) or Ca^{2+} -CaM [1]. Upon Ca^{2+} binding, CaM binds to various target proteins and regulates their function, thereby transducing Ca^{2+} signals. These target proteins include myosin light chain kinase (MLCK), phosphodiesterase (PDE), adenylyl cyclase (AC), Ca^{2+} /CaM-dependent kinase (CaMK), protein phosphatase calcineurin and nitric-oxide synthase (NOS), Ca^{2+} -activated K^+ channels, L-type Ca^{2+} channels (LTCC), ryanodine receptors (RyR) etc [2–5]. Notably, ApoCaM also binds to many proteins (e.g. ion channels, actin-binding proteins, cytoskeletal and membrane proteins, enzymes, receptors [1,6–9], which may pre-localize ApoCaM near Ca^{2+} -CaM targets for

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locally mediated activation (as in ion channels). Functional effects of CaM on channel gating, Ca^{2+} release, cell cycle progression and cardiac hypertrophy has been demonstrated [10–13].

The amount of CaM is typically about 0.1% of the total cellular proteins and total cellular CaM concentration ($[\text{CaM}]_{\text{total}}$) has been reported to be within the range of 2–25 μM depending on different tissues [14]. In cardiac myocytes, $[\text{CaM}]_{\text{total}}$ is about 6 μM [15–17]. However under basal conditions (i.e. at low $[\text{Ca}^{2+}]_i$) it is less clear how much ApoCaM is already prebound and how much is free ($[\text{CaM}]_{\text{free}}$) and this may differ in different cell types. Experiments with serum-deprived Swiss 3T3 fibroblasts first indicated that the majority of CaM was freely diffusible, but the CaM was then immobilized in response to stimulation by serum [18]. In HEK cells, around half of the CaM was free [19] while most of the CaM was bound to the cellular structures in smooth muscle cells [20]. This may reflect variability of the state of CaM in different cell types. Striated muscle cells differ from other cell types regarding organization of intracellular structure and physiological function. Persechini and colleagues designed fluorescent indicator proteins with various Ca^{2+} -CaM binding affinities that work by changes in fluorescence resonance energy transfer (FRET) [21]. Using one of these sensors in adult ventricular myocytes, our lab concluded that $[\text{CaM}]_{\text{free}}$ might be very low in cardiac myocytes, because a sensor with Ca^{2+} -CaM $K_d=45$ nM could not compete for endogenous CaM in myocytes [22]. However, more direct estimates of $[\text{CaM}]_{\text{free}}$ in cardiac myocytes have not been performed.

In resting conditions, cytosolic $[\text{Ca}^{2+}]_i$ is very low (100–150 nM) and most CaM is ApoCaM. Some ApoCaM may already be bound to protein targets either as structural parts of the protein or awaiting local activation by Ca^{2+} binding. The remaining ApoCaM is free and would be subject to more global competition among CaM binding sites. When $[\text{Ca}^{2+}]_i$ is high, more CaM exists as Ca^{2+} -CaM. Upon Ca^{2+} binding, targets with prebound CaM may be activated immediately, while targets lacking prebound ApoCaM must compete for free Ca^{2+} -CaM in the cytosol. The proteins that could bind Ca^{2+} -CaM are much more numerous than total CaM [21,23] and they have different affinities for Ca^{2+} -CaM. At saturated $[\text{Ca}^{2+}]_i$ the $[\text{CaM}]_{\text{free}}$ required for half maximal activation of MLCK is ~ 1 nM [24] and for CaMKII is ~ 50 nM [25], while those for calcineurin, PDE and plasma membrane Ca^{2+} -ATPase (PMCA) are 0.1 nM [26], 0.1–1 nM [27], and 5 nM [28], respectively. Binding affinity may be important during the competition among target proteins to bind Ca^{2+} -CaM. As a result, higher affinity targets can capture Ca^{2+} -CaM from lower affinity targets and shape the time courses of target responses in the cell [29]. At the same time, Ca^{2+} -CaM can change its subcellular location according to the gradients of $[\text{Ca}^{2+}]_i$ and Ca^{2+} -CaM binding proteins with different affinities. Changes of $[\text{Ca}^{2+}]_i$ have been shown to favor CaM redistribution to the nucleus in smooth muscle and pancreatic cells [30,31]. This stimulus dependent redistribution of CaM to the nucleus has also been observed in neurons [32,33], but Ca^{2+} -CaM redistribution in cardiac myocytes has not been investigated yet.

Here we aim to measure resting $[\text{CaM}]_{\text{free}}$ in adult cardiomyocytes and how CaM moves upon changes in $[\text{Ca}^{2+}]_i$. We found that at resting $[\text{Ca}^{2+}]_i$ CaM is relatively localized at Z-lines and the $[\text{CaM}]_{\text{free}}$ is between 50–75 nM. Elevation of $[\text{Ca}^{2+}]_i$ favors CaM translocation to the nucleus and Z-lines. In addition, high $[\text{Ca}^{2+}]_i$ greatly slowed CaM dissociation rate.

2. Materials and methods

2.1. Cell isolation

All procedures were performed in accordance with Guide for the care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Rabbit myocytes were isolated as previously described [34]. Briefly, rabbits were anaesthetized by I.V. injection of pentobarbital sodium (50–70 mg kg^{-1}) and rabbits were anaesthetized by I.P. injection of

Nembutal (~1 mg g⁻¹). Hearts were excised quickly and placed on a Langendorff perfusion apparatus. Hearts were perfused for 5–7 min with nominally Ca²⁺-free Tyrode solution, and then perfusion was switched to the same solution containing 1 mg ml⁻¹ collagenase. When the heart became flaccid, the ventricles were dispersed and filtered. The cell suspension was washed several times with a gradual increase in the [Ca²⁺] up to 2 mM. Normal Tyrode's (NT) solution contained (in mM): NaCl 140, KCl 4, MgCl₂ 1, CaCl₂ 2, glucose 10, HEPES 5, pH 7.4. All experiments were carried out at room temperature.

2.2. Cell permeabilization

For cells that were originally suspended in NT solution, first we let them settle down for 20 min, and then changed the bath solution to relaxation solution for 2 min. Then cells were permeabilized with saponin (50 µg/ml) for 20 s immediately followed by internal solution wash-in. Relaxing and permeabilizing solution (mM): EGTA 0.1, HEPES 10, K-aspartate 120, MgCl₂ 1 free, ATP 5, reduced glutathione 10, phosphocreatine di-tris 5 and saponin 50 µg/ml (only in permeabilizing solution), pH 7.4; Skinned fiber solution was the same except that free [Ca²⁺] was adjusted to 100 nM (or as noted) and 8% dextran was added to prevent myofilament lattice swelling upon permeabilization. The pH was adjusted to 7.2. MaxChelator was used to calculate reagents.

2.3. Patch clamp

Bath solution is 2 mM Ca²⁺ NT. Pipette solution includes (mM): KCl 140, MgCl₂ (to attain 1 mM free [Mg²⁺]_i), glucose 10, Tris-ATP 5, Hepes 10, NaCl 10 and F-CaM 1 µM. No EGTA was included. The pipette resistance is 2.5 MΩ and or access resistance is 4–10 MΩ.

2.4. Immunocytochemistry and Western Blotting

The immunocytochemistry was according to [32] with minor modifications. Briefly, the myocytes were fixed with 2% paraformaldehyde (with 4 mM EGTA) for 20 min and washed with PBS twice for 5 min each. Then they were permeabilized with 0.5% triton in PBS for 20 min and washed with PBS twice for 5 min each. The cells were blocked with 2% BSA for 1 hr and incubated with primary antibody for 2 hr before another blocking for 30 min and secondary antibody incubation for 1 hr. Images were acquired by confocal microscopy. Western blotting was done as described [35].

2.5. Reagents and antibodies

Alexa Fluor 488 conjugate CaM (F-CaM), non-fluorescent CaM and Di-8-ANEPPS were from Molecular Probes. Alexa Fluor 488 is a 643 Da small molecule conjugated to the N terminal of CaM molecule. Anti-CaM primary antibody was from Upstate Biotechnology and 2,3-butanedione monoxime (BDM) was from Fluka.

2.6. Confocal imaging

Both F-CaM and Di-8-ANEPPS signals were excited by argon laser at 488 nm wavelength. The emission wavelength was 500–530 nm for F-CaM and more than 600 nm for Di-8-ANEPPS signals. This setting ensured that these two signals have little cross talk. For the immunocytochemistry image, CaM signal was detected by secondary antibody at excitation wavelength of 488 nm and emission wavelength of more than 500 nm. Di-8-ANEPPS (10 µM) was loaded for 30 min and excess indicator was washed out for 15 min. The image was recorded via confocal microscopy.

2.7. Statistical analysis

Data are presented as mean $\pm$ SEM with significance ($p < 0.05$) determined using unpaired 2-tailed Student's t test.

3. Results

3.1. CaM localization in cardiac myocytes

CaM localization in cardiac myocytes was assessed by three approaches: immunocytochemistry (Fig 1A), F-CaM wash-in in permeabilized myocytes (Fig 1B) and patch pipette dialysis F-CaM into intact cells (Fig 1C). CaM localization showed a similar striated pattern in all three cases. To test whether this CaM concentration is at Z-lines vs. M-lines, cells were stained with the membrane indicator Di-8-ANEPPS to identify transverse-tubules which occur at the Z-lines. Then cells were permeabilized, with subsequent F-CaM wash-in for 5–10 min. This time range gave both clear Di-8-ANEPPS and F-CaM signals at the same time. Longer F-CaM wash-in caused gradual loss of Di-8-ANEPPS signal, while shorter F-CaM wash-in time gave weaker F-CaM signals. Fig 1D shows simultaneous 2-D images for F-CaM and Di-8-ANEPPS. The line plot in Fig 1E shows that Di-8-ANEPPS and F-CaM signals overlapped spatially and that the distance between striations was about 1.9 μm (the sarcomere length). This confirmed that CaM is relatively concentrated at Z-lines (e.g. vs. M-lines). We also tested for fluorescence signal cross-talk by obtaining either F-CaM or Di-8-ANEPPS signal at the two emission wavelengths (500–530 nm & >600 nm) separately from single reagent staining at the same settings. The F-CaM signal detected at >600 nm is less than 5% of that at 500–530 nm, and no Di-8-ANEPPS signal was detectable at 500–530 nm. This confirms that the F-CaM and Di-8-ANEPPS signals do not suffer appreciable cross contamination.

3.2. F-CaM wash-in right after permeabilization is a two-phase process

Fig 2 shows the wash-in of F-CaM (bath $[\text{F-CaM}] = 100 \text{ nM}$; $[\text{Ca}^{2+}]_i = 100 \text{ nM}$) immediately after permeabilization. The time course of the myocyte F-CaM signal is shown after subtraction of background fluorescence and the fluorescence contributed by the free 100 nM F-CaM (using the signal just outside the cell). Thus, this signal represents binding of F-CaM to cellular sites (not the rise in free $[\text{F-CaM}]$ in the cell, which rises much faster in control experiments, see Fig 7B inset). This curve was fit by a double exponential association equation with fast and slow phases (Fig 2). The fast phase is likely to be F-CaM which rapidly binds to unoccupied CaM binding sites and slow phase probably reflects (in large part) F-CaM that competes at sites where endogenous CaM is already bound and gradually replaced. From here the fast and slow phase time constants (τ_f and τ_s) and amplitudes (Y_f and Y_s) are obtained: $\tau_f = 34.2 \pm 2.6 \text{ s}$, $\tau_s = 14.8 \pm 1.9 \text{ min}$ (26-fold different), $Y_f = 7.4 \pm 0.7$, $Y_s = 26.2 \pm 2.5$ (4-fold different). While we do not attribute real binding kinetic significance to the τ_f (i.e. it is complicated by wash-in and diffusional variation), the 26-fold difference in τ_f vs. τ_s makes it rather easy to separate these two phases.

3.3. Measurement of $[\text{CaM}]_{\text{free}}$

We used these type of experiments to design two modified null-point titration methods to estimate endogenous $[\text{CaM}]_{\text{free}}$ in intact myocytes. Method 1 (Fig 3A) uses washed-in of different $[\text{F-CaM}]$ immediately after permeabilization. The F-CaM signal 30 s after wash-in was recorded (Fig 4A). Fluorescence (F) after 30 s reflects almost exclusively the fast phase amplitude (i.e. 3% saturation of slow phase, but 60% saturation of fast phase). If $[\text{F-CaM}]$ is the same or lower than endogenous myocyte $[\text{CaM}]_{\text{free}}$, the fast phase will be small (Fig 3A). However, when $[\text{F-CaM}]$ is higher than endogenous $[\text{CaM}]_{\text{free}}$, during wash-in, the fast phase

F should increase progressively (Fig 3A and 4A). The transition is the null point $[\text{CaM}]_{\text{free}}$, and this transition occurred at about 50 nM F-CaM.

Method 2 (Fig 3B) used preincubation of permeabilized myocytes with different $[\text{CaM}]$ (non-fluorescent) for 20 min followed by F-CaM (100 nM) wash-in. There is a balance between the bound and unbound CaM. Thus, if the cells are preincubated with a $[\text{CaM}]$ that is lower than the intact myocyte $[\text{CaM}]_{\text{free}}$, some endogenous CaM will dissociate creating more unoccupied CaM binding sites. This will increase Y_f measured later upon F-CaM (and vice versa). But if the actual myocyte $[\text{CaM}]_{\text{free}}$ is used to pre-incubate the cells, the measured Y_f will be the same as that obtained by immediate wash-in of 100 nM F-CaM upon permeabilization (Y_{f1} ; without non-F-CaM preincubation). This is the null point $[\text{CaM}]_{\text{free}}$ (i.e. $Y_f - Y_{f1} = 0$ in Fig 4B) Figure 3B and C illustrate schematically and with experimental data the three possible cases for Method 2. Pooled data from experiments like this (Fig 4B) show that the null point is 70–75 nM $[\text{CaM}]_{\text{free}}$ in intact myocytes. Thus, both methods agree that $[\text{CaM}]_{\text{free}}$ in intact resting ventricular myocytes is in the range of 50–75 nM, which is only about 1% of the total cellular CaM [15–17].

If most of the CaM in the resting myocyte is already bound to cellular constituents, we might expect it to be relatively well retained upon abrupt permeabilization. To examine this we compared the amount of CaM in intact vs. abruptly permeabilized myocytes using Western blotting. The same amounts of cells were spun down rapidly (10 s in a hand-crank centrifuge) either with or without permeabilization by a 20 s exposure to saponin (Fig 4C). CaM data was quantified by densitometric scans and normalized to levels of the intra-SR protein calsequestrin (CSQ), which should not be lost upon sarcolemmal permeabilization. Figure 4D shows that there was no significant loss of CaM in this short time after permeabilization (although total protein in the pellet was reduced by ~20%). This is consistent with our null point results indicating that cytosolic $[\text{CaM}]_{\text{free}}$ is very low and >95% of myocyte CaM is bound to cellular constituents in resting intact ventricular myocytes (at 100 nM $[\text{Ca}^{2+}]_i$).

3.4. High $[\text{Ca}^{2+}]_i$ favors CaM bindings, especially at nucleus and Z lines

Since increasing $[\text{Ca}^{2+}]_i$ is expected to increase the CaM affinity for its cellular targets we also elevated $[\text{Ca}^{2+}]$ to determine if the F-CaM binding would increase and if its cellular distribution or location would change. Both permeabilized and intact cells were tested with similar qualitative results. In permeabilized myocytes, F-CaM (60 nM) was washed-in with $[\text{Ca}^{2+}]_i$ clamped at 100 nM or 500 nM (Fig 5A). After 40 min wash-in, CaM binding reached a plateau. Overall, more F-CaM was bound with 500 nM $[\text{Ca}^{2+}]_i$ (Fig 5A–B) and this was reversible (not shown). A longitudinal plot profile (Fig 5B) shows the sarcomeric pattern and that F-CaM at the Z-line increased more than nearer the center of the sarcomere (M-line). For simplicity, we considered only three representative regions of interest (Z-line, M-line and nucleus). Fluorescence levels were normalized to M line at 100 nM $[\text{Ca}^{2+}]_i$ (F_M (100 nM)) and per unit area. At 100 nM $[\text{Ca}^{2+}]_i$ the $[\text{F-CaM}]$ at the Z-line was ~50% higher than at the M-line or nucleus (Fig 5C). Raising $[\text{Ca}^{2+}]_i$ to 500 nM enhanced CaM binding everywhere, but the relative increase was greatest in the nucleus > Z line > M line (Fig 5D).

This shift was also apparent in myocytes under more intact conditions (Fig 6), where F-CaM (1 μM) was dialyzed more slowly in via a patch-clamp pipette (access resistance 4–10 M Ω). With 10 mM $[\text{Na}^+]$ in the pipette solution ($[\text{Ca}^{2+}]_i$ was nominally 0 mM without EGTA) membrane potential (V_m) was clamped initially at –80 mV for several minutes to keep $[\text{Ca}^{2+}]_i$ low. F-CaM gradually entered the cell and showed the characteristic striated pattern within 10 min. When the myocyte was depolarized to +50 mV (to drive Ca^{2+} entry via $\text{Na}^+/\text{Ca}^{2+}$ exchange) the cell shortened by ~10%, consistent with elevated $[\text{Ca}^{2+}]_i$. The F-CaM signal also increased to a new plateau (Fig 6B), which likely reflects enhanced F-CaM binding in the cell at higher $[\text{Ca}^{2+}]_i$. In contrast, beat to beat Ca^{2+} transients induced by short voltage

clamp depolarizations to 0 mV (for 100 ms at 1 Hz) did not appreciably alter the trajectory of the rise in myocyte $[F\text{-CaM}]$ (Fig 6B). One can also see enhanced F-CaM binding in the nucleus when $[Ca^{2+}]_i$ was elevated at +50 mV. Notably, in both Figures 5 and 6 there is effectively an infinite source of F-CaM. However, the clear implication is that in the intact cell (with a finite pool of CaM) there would be net spatial redistribution of CaM as $[Ca^{2+}]_i$ rises.

3.5. F-CaM calibration

To determine how much F-CaM was bound and how much endogenous CaM was displaced from binding sites, F-CaM was calibrated in vitro and in vivo. For in vitro calibration, different $[F\text{-CaM}]$ was used in internal solution without or with 50 mg/ml BSA (to mimic the protein-rich intracellular environment). F-CaM fluorescence increased linearly with $[F\text{-CaM}]$ and BSA only slightly reduced F-CaM apparent fluorescence (Fig 7A; slope reduced by 10%). For in vivo calibration (Fig 7B), the cells were permeabilized and preincubated with non-fluorescent CaM (10 μM) for 10 min ($[Ca^{2+}]_i = 100 \text{ nM}$) to saturate rapidly available CaM binding sites. This was followed by wash-in with different $[F\text{-CaM}]$ in the continued presence of 10 μM CaM for only 1 min. The inset is an example of rapid rise of intracellular fluorescence during free F-CaM wash-in. Bath fluorescence and cellular fluorescence were simultaneously recorded (per unit area). Similar to the in vitro calibration, F-CaM fluorescence increased linearly with $[F\text{-CaM}]$ and was only slightly lower in the cellular environment than in the bath. This relationship was used to calibrate the $[F\text{-CaM}]$ in images where the bath $[F\text{-CaM}]$ is known and the cellular fluorescence is measured (as in Fig 2).

Fig 7C shows the bound $[F\text{-CaM}]$ ($[F\text{-CaM}]_{\text{bound}}$ in $\mu\text{mol/l}$ cytosol) obtained by subtracting cell background fluorescence (from the image just prior to F-CaM wash-in) and the 60 nM basal F-CaM fluorescence inside the cell (i.e. the 60 nM bath fluorescence corrected by the ratio of slopes from the in vivo calibration Fig 7B). We found that F-CaM (60 nM) wash-in for 40 min could replace 4–5 μM endogenous CaM (Fig 7C). This is more than half of the total CaM in these myocytes (6 μM ; [17]). This also confirms that at 100 nM $[Ca^{2+}]_i$ and 60 nM $[CaM]_{\text{free}}$ (which we inferred as the endogenous value in intact myocytes; Fig 3–4) one can obtain levels of total CaM comparable to that in the intact myocyte. Of course, some fraction of endogenous bound CaM may not be displaceable with F-CaM over this time, and that could account for the slightly lower value vs. intact cells. There may also be a slow loss of some CaM targets from the myocyte over this longer time frame than in the experiments in Fig 4C–D. Nevertheless, the relatively good quantitative agreement of these separate results gives us confidence that our $[CaM]_{\text{free}}$ estimates are correct.

3.6. Higher $[Ca^{2+}]_i$ decreases CaM dissociation rate

From the above we know that 4–5 μM CaM can be displaced by F-CaM, and we assumed that the slow phase of F-CaM binding in Fig 3 was due to slow dissociation of endogenous CaM at physiological diastolic $[Ca^{2+}]_i$. To further test that assumption we measured the dissociation rate of F-CaM after a steady plateau was obtained. Fig 8 shows that wash-in of 60 nM F-CaM (at 100 nM $[Ca^{2+}]_i$) resulted in binding with a time constant τ_b of $11.1 \pm 1.5 \text{ min}$. At the plateau $[Ca^{2+}]_i$ was elevated to 500 nM at the same time as F-CaM was withdrawn. This allowed washout of F-CaM without appreciable loss of bound F-CaM. Upon reduction of $[Ca]_i$ back to 100 nM there was F-CaM dissociation with a time constant τ_d of $11.7 \pm 1.8 \text{ min}$, similar to that seen during binding (τ_b). This indicates that our assumption that the slow phase of F-CaM binding was indeed limited by the rate of loss of endogenous CaM from its binding sites. This figure also indicates that higher $[Ca^{2+}]_i$ decreases CaM dissociation rate, presumably by increasing CaM affinity to targets.

4. Discussion

Using F-CaM and two modified null point titration methods, we measured the $[CaM]_{free}$ in resting adult ventricular myocytes to be 50–75 nM. This was consistent with CaM Western blotting showing little loss of CaM immediately after permeabilization. This $[CaM]_{free}$ is only about 1% of the total CaM in the myocyte, indicating that even at resting $[Ca^{2+}]_i$ (100 nM) most CaM is bound to cellular constituents. The $[CaM]_{free}$ is especially high at the Z-line (or transverse tubule) and upon elevation of $[Ca^{2+}]_i$ more CaM binds to cell constituents, especially in the nucleus. In F-CaM the CaM is labeled with Alexa Fluor 488 (a small molecule, MW 643 Da) and is not expected to affect the conformation of CaM (and its fluorescence is not sensitive to Ca^{2+} binding to CaM). Indeed, F-CaM gave similar localization to endogenous CaM. Thus, we think that F-CaM is a good marker for CaM in our studies. The CaM localization at the Z line in rabbit ventricular myocytes was also found in other species (e.g. adult rat and mouse cardiac myocytes, not shown).

4.1 Implications of low $[CaM]_{free}$ in myocytes

The $[CaM]_{free}$ is critical in CaM physiology because it influences the competition between different CaM target proteins. Indeed, the low $[CaM]_{free}$ in myocytes means that there is likely to be dynamic competition among Ca^{2+} -CaM targets in the cell, a point already suggested by work in other cell types (Persechini & Stemmer, 2002). Thus, even when $[Ca^{2+}]_i$ and $[Ca^{2+}$ -CaM] are elevated globally in myocytes, targets with higher Ca^{2+} -CaM affinities ($K_d \leq 1$ nM, e.g. calcineurin, PDE, MLCK) may be preferentially activated vs. those with lower affinity ($K_d > 10$ nM, e.g. CaMK, AC), especially as $[CaM]_{free}$ would progressively decline as more free Ca^{2+} -CaM bound to targets..

Conversely, the large amount of CaM already bound in myocyte sites under conditions where most CaM is expected to be ApoCaM, may indicate that ApoCaM is prebound near sites that are Ca^{2+} -CaM targets. For example, it is known that CaM is prebound to both the cardiac RyR and LTCC, where Ca^{2+} -CaM inhibits RyR opening and causes Ca^{2+} -dependent inactivation, respectively [10,11]. At a stoichiometry of one CaM per RyR monomer this would constitute nearly 1 μ M CaM bound to RyR (and ~50 nM for LTCC). These channel proteins are not especially concentrated in myocytes, and it seems likely that there are other Ca^{2+} -CaM target proteins (or their neighbors) which prebind and thus localize CaM near targets. Just the RyR sites alone would account for 15–20% of the total CaM in myocytes. This pre-bound CaM may poise ApoCaM at sites ready for functional target activation as Ca^{2+} -CaM when local $[Ca^{2+}]$ rises. In the case of the cardiac junction where both LTCC and RyR reside, CaM localization may facilitate direct Ca^{2+} -CaM effects on Ca^{2+} current and SR Ca^{2+} release, and also enhance the ability of local CaM to activated local CaMKII, which is also associated with these same proteins [36–38]. The local $[Ca^{2+}]_i$ in this cleft also gets very high during Ca^{2+} channel fluxes. This sort of localization of CaM may thus enhance the ability of lower Ca^{2+} -CaM affinity targets like CaMKII to compete with higher affinity targets and be activated.

The $[CaM]_{free}$ may vary widely in different cell types, the population of ApoCaM and Ca^{2+} -CaM targets and the $[Ca^{2+}]_i$. One report demonstrated that particulate CaM (storage CaM) is more abundant in non-cancerous vs. cancerous cells [39]. ApoCaM was suggested to serve as storage reservoir for CaM, mobilized when cells are stimulated or enter the cell cycle [1]. In HEK 293 cells, a novel fluorescent indicator suggested that available $[CaM]$ was 8.8 μ M, or ~30% of total CaM in those cells [19]. Biochemical separation of particulate vs. soluble cell fractions [14] or long-term triton extraction of smooth muscle [20] would suggest only a moderate fraction of CaM (~50%) being strongly bound to cell structures (at low $[Ca^{2+}]$). Superficially, our results would appear to be at odds with these findings, but we think the difference may be semantic. That is, our $[CaM]_{free}$ results are based on known $[F-CaM]_{free}$ in the bath (with which the myocyte is equilibrated) and probably reflect truly unbound CaM that

is free to diffuse. The available [CaM] [19], CaM which is released by extensive triton/glycerol extraction [20] or in supernatant fractions [14] could still have been largely interacting (at least weakly) with cellular proteins and still be included in that category. Notably, Tansey *et al.* [20] found that permeabilization of smooth muscle by β -escin (similar to saponin used here) allowed almost complete loss of a 40 kDa dextran, but no significant loss in CaM over 45 min. They also found that the apparent CaM diffusion in smooth muscle cells was extremely slow, consistent with strong binding of a majority of CaM. Similar, the elegant indicator-based estimates of “available CaM” (which was functionally defined as such [19]) do not exclude the possibility that some CaM sensed by the indicator is also involved in other interactions. Moreover, using Ca^{2+} -CaM sensors, that same group detected a maximal free [Ca^{2+} -CaM] of 45 nM at maximal [Ca^{2+}]_i in HEK cells [21], which could conceivably better reflect more truly unbound CaM (because binding to that higher affinity sensor may preclude binding to other proteins). In conclusion, we believe that our measurements of [CaM]_{free} in myocytes are correct and that these low values of free [CaM] and [Ca^{2+} -CaM] emphasize the importance of CaM target competition and/or localization effects discussed above.

4.2 CaM affinity for cellular sites

At 500 nM [Ca^{2+}]_i the CaM dissociation rate from binding sites in the cell was negligible vs. that at 100 nM [Ca^{2+}]_i. We suppose that this is a result of the much higher affinity of Ca^{2+} -CaM for its targets vs. a presumably weaker affinity of ApoCaM (which may predominate at 100 nM [Ca^{2+}]_i). On the other hand, the 11 min time constant for F-CaM washout at 100 nM [Ca^{2+}] suggests a rather high affinity (sub-nM K_d for CaM if the on-rate is diffusion-limited). However, the complicated cellular environment with many binding sites complicates quantitative inferences about binding kinetics (see also below).

In preliminary F-CaM washout experiments (like in Fig 8) we did not include non-fluorescent CaM in the washout solution, but in that case a very large amount of F-CaM remained bound even after 40 min washout. This could be due to the absence of competition by CaM. That is, if F-CaM comes off one site it may rebind again (there or at a neighboring site). However, when 60 nM CaM was included in the washout phase (as in Fig 8), the exogenous CaM could compete with rebinding of F-CaM. Even with 60 nM CaM in the F-CaM washout there was still F-CaM left (plateau in Fig 8 is at ~36%). This may be the same “rebinding” issue as above or could also reflect sites with extremely low off-rates. Indeed, it is important to appreciate that we are assessing overall CaM binding to many different sites, and the properties we infer apply to this ensemble of CaM binding sites, rather than any specific site.

4.3 Translocation of CaM in myocytes

Nuclear CaM functions in various ways. Nuclear CaMKII in many cell types is consistent with the hypothesis of CaM-directed phosphorylation of nuclear proteins [40–42]. In cardiac myocytes, CaM overexpression increased the autonomous activity of CaMKII, which regulates atrial natriuretic factor (ANF) gene expression and induces hypertrophy [13,43]. CaM and CaMKII both associate with inositol triphosphate receptor at the nuclear envelope, where these proteins function together in cardiac excitation-transcription coupling [44]. CaM was also shown to compete with myocyte enhancer factor-2 (MEF2) for binding Histone Deacetylase 5 to activate transcription [45]. In neurons, activation of Ca^{2+} channels cause mobilization and translocation of CaM from the cytoplasm to the nucleus, which is important for the rapid phosphorylation of cAMP response element binding protein (CREB) [32,46]. Thus CaM redistribution to the nucleus may be physiologically important.

Our previous work has shown that endothelin-1 stimulates CaM translocation to the nucleus, without a detectable elevation of [Ca^{2+}]_i in resting ventricular myocytes, but that normal myocyte Ca^{2+} transients do not cause appreciable nuclear translocation [44]. Here we

complement that data to show that sustained elevation of $[Ca^{2+}]_i$ to 500 nM clearly shifts CaM into the nucleus. The implication is that chronic $[Ca^{2+}]_i$ elevation is required for Ca^{2+} -dependent CaM nuclear import, but also other pathways that do not elevated global $[Ca^{2+}]_i$ can also cause translocation. Given the apparently low $[CaM]_{free}$ and implied low mobility, it is possible that the CaM translocation to the nucleus is together with some protein partner that directs it there. This was also the conclusion drawn in neurons where signaling induced nuclear import of CaM was in association with other protein partners [32,33].

In the present study in permeabilized (or pipette dialyzed) myocytes there is an unlimited reservoir of 60 nM CaM in the bath (or higher $[CaM]$ in the pipette). This may make the F-CaM redistribution more apparent, but similar qualitative shifts must occur in the intact cell where CaM is in limited supply (although the kinetics and extent would be different). Elevated $[Ca^{2+}]_i$ increases CaM binding everywhere, although nuclear CaM increased the most, followed by Z lines, while M lines were the least affected. The high $[CaM]$ at the Z-line is not especially surprising, because at least several targets are known to be there (e.g. RyR, LTCC, CaMKII, calcineurin, NOS), some of which are also known to bind ApoCaM. However, our method here cannot discriminate a particular target. There certainly may also be redistribution within these regions as local Z-line CaM may move to nearby Ca^{2+} -CaM targets when $[Ca^{2+}]_i$ is elevated.

In conclusion, CaM location and response to different $[Ca^{2+}]_i$ in cardiac myocytes are consistent with both physiological and pathological functions of CaM. Changes of CaM location may be an indicator of changes of cellular function.

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Fig 1. CaM is relatively localized on Z-lines. (A) Immunofluorescence of endogenous CaM. (B) F-CaM wash-in in permeabilized ventricular myocytes. (C) F-CaM dialyzed in intact myocytes via patch pipette (at elevated $[\text{Ca}^{2+}]_i$). (D) F-CaM wash-in in Di-8-Anepps loaded cells. Images were taken simultaneously and merged at right. (E) The same region of interest (ROI) was chosen on both F-CaM and Di-8-ANEPPS images. Plot profile shows overlap of F-CaM and Di-8-ANEPPS signals.

Fig 2.

F-CaM “wash-in” right after myocyte permeabilization. 100 nM F-CaM was washed-in immediately after cell permeabilization (20 s saponin application) for 40 min. Background fluorescence and fluorescence due to $[CaM]_{free}$ in the cell have been subtracted already. F-CaM binding is fit by a two-phase exponential: a fast phase (blue) and slow phase (green) using $Y = Y_f(1 - \exp(-t/\tau_f)) + Y_s(1 - \exp(-t/\tau_s))$. (N=8 cells). The inset shows an expanded time scale for the first 4 s.

(A) Method 1

(B) Method 2

Fig 3.

Evaluation of $[CaM]_{free}$ using two null-point titration methods. (A) Principle of method 1. Different concentrations of F-CaM are washed-in immediately after permeabilization and different amplitudes of fast phase are seen. If $[F-CaM] \leq [CaM]_{free}$, then there will be no or little fast phase. If $[F-CaM] > [CaM]_{free}$, then a rapid increase of fast phase amplitude will be seen. The transition $[F-CaM]$ (null point) is $[CaM]_{free}$ in the intact cell (see Fig 4A). (B) Principle of method 2. Different non-fluorescent $[CaM]$ are preincubated for 20 min after permeabilization. If the $[CaM] < [CaM]_{free}$, then more endogenous CaM will dissociate and a larger fast phase will be seen upon F-CaM addition (vs. acute permeabilization) and vice versa. If $[CaM] = [CaM]_{free}$, then a same fast phase will be seen as F-CaM wash-in immediately after

permeabilization (null point; see Fig 4B). (C) Illustration of different [CaM] preincubations followed by 100 nM F-CaM wash-in. In A and B Filled circles represent bound CaM sites, while open circles represent unoccupied CaM binding sites; Green circles represent F-CaM binding in the fast phase.

Fig 4.

Summary of two null point methods and confirmation by Western blotting. (A) fluorescence at 30 s after permeabilization (Y_f) as a function of $[F\text{-CaM}]$, implying that $[CaM]_{\text{free}}$ is about 50–75 nM (N=6 cells). (B) Average differences between Y_f and Y_{f1} (Y_f upon acute permeabilization with the same $[F\text{-CaM}]$) as a function of $[CaM]$ during 20 min pre-incubation after permeabilization. The $[CaM]_{\text{free}}$ implied for the intact resting myocyte is 50–75 nM. (N=6 cells). (C). Same amount of cells were permeabilized or not and immediately spun down, with the same volume of lysis buffer for Western blotting analysis. Same volume of cell lysates were loaded. CSQ is as control (representative of five experiments). (D) pooled data of experiments in C.

Fig 5.

Higher $[Ca^{2+}]_i$ favors CaM binding in permeabilized cells, especially at nucleus and Z lines. F-CaM 60 nM was washed-in in permeabilized cells for 40 min at 100 nM $[Ca^{2+}]_i$ and 500 nM $[Ca^{2+}]_i$ respectively. (A) Images after 40 min of 60 nM [F-CaM] wash-in. (B) Plot profile showed CaM binding at Z-lines and M-lines. (C) Bar graph compared fluorescence (normalized to M-lines at 100 nM $[Ca^{2+}]_i$) at M-lines, Z-lines and nucleus when $[Ca^{2+}]_i$ is 100 nM and 500 nM. Regions of interest were centered on the Z-lines ($\sim 1 \mu$ m long) and regions between Z-lines (M-Lines) or from the entire nucleus (normalized per unit area). (D) Relative increase of F-CaM binding at $[Ca^{2+}]_i$ 500 nM over 100 nM at M-lines, Z-lines and nucleus. * $P < 0.05$

Fig 6.

Higher $[Ca^{2+}]_i$ favors CaM binding in intact cells. 1 μ M CaM was included in the dialyzing patch pipette, and holding potential was switched from -80 to $+50$ mV to activate Ca^{2+} influx via NCX. (A) Representative images (enlarged insets are centered on the pipette in i, but the upper nucleus in ii–iv). (B) Time course of CaM binding during 20 min of quiescence at -80 mV ($N=9$ cells), followed by 20 min at either $+50$ mV ($N=5$) or at -80 mV with 100 ms pulses to 0 mV at 1 Hz ($N=4$).

Fig 7.

F-CaM calibration. (A) In vitro calibration. The fluorescence of different $[F-CaM]$ in internal solution with or without BSA 50 mg/ml was recorded by confocal microscopy and linear regression is shown ($N=11$ experiments). (B) In vivo calibration. Permeabilized myocytes were prebound with 10 μM CaM for 20 min to saturate the unoccupied CaM binding sites with non-fluorescent CaM, followed by wash-in of different $[F-CaM]$. Fluorescence was taken 1 min after F-CaM wash-in. ($N=6$ cells). Inset shows the time course of diffusion of 100 nM F-CaM into the cell. (C) Total bound F-CaM (μM) for Fig 6. was plotted according to this calibration ($N=6$ cells at 100 nM or 4 cells at 500 nM).

Fig 8.

Higher $[Ca^{2+}]_i$ decreased CaM dissociation rate. F-CaM 60 nM was washed-in for 40 min and washed-out in the presence of 60 nM CaM at 500 nM $[Ca^{2+}]_i$ for 20 min followed by 100 nM $[Ca^{2+}]_i$ for another 40 min. (N=6 or 5 cells).