Voltage Dependence of Cardiac Excitation-Contraction Coupling

Unitary Ca\(^{2+}\) Current Amplitude and Open Channel Probability

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Abstract—Excitation–contraction coupling in cardiac myocytes occurs by Ca\(^{2+}\)-induced Ca\(^{2+}\) release, where L-type Ca\(^{2+}\) current evokes a larger sarcoplasmic reticulum (SR) Ca\(^{2+}\) release. The Ca\(^{2+}\)-induced Ca\(^{2+}\) release amplification factor or gain (SR Ca\(^{2+}\) release/ICa) is usually assessed by the V\(_m\) dependence of current and Ca\(^{2+}\) transients. Gain rises at negative V\(_m\) as does single channel ICa (i\(_{Ca}\)), which has led to the suggestion that the increases of i\(_{Ca}\) amplitude enhances gain at more negative V\(_m\). However, ICa=NP\(_o\)×i\(_{Ca}\) (where NP\(_o\) is the number of open channels), and NP\(_o\) and i\(_{Ca}\) both depend on V\(_m\). To assess how i\(_{Ca}\) and NP\(_o\) separately influence Ca\(^{2+}\)-induced Ca\(^{2+}\) release, we measured ICa and junctional SR Ca\(^{2+}\) release in voltage-clamped rat ventricular myocytes using “Ca\(^{2+}\) spikes” (confocal microscopy). To vary i\(_{Ca}\) alone, we changed [Ca\(^{2+}\)]o, rapidly at constant test V\(_m\) (0 mV) or abruptly repolarized from +120 mV to different V\(_m\). To vary NP\(_o\), we altered Ca\(^{2+}\) channel availability by varying holding V\(_m\). Reducing either i\(_{Ca}\) or NP\(_o\), alone increased excitation–contraction coupling gain. Thus, increasing i\(_{Ca}\) does not increase gain at progressively negative test V\(_m\). Such enhanced gain depends on lower NP\(_o\) and reduced redundant Ca\(^{2+}\) channel openings (per junction) and a consequently smaller denominator in the gain equation. Furthermore, modest i\(_{Ca}\) (at V\(_m\)=0 mV) may still effectively trigger SR Ca\(^{2+}\) release, whereas at positive V\(_m\) (and smaller i\(_{Ca}\)), high and well-synchronized channel openings are required for efficient excitation–contraction coupling. At very positive V\(_m\), reduced i\(_{Ca}\) must explain reduced SR Ca\(^{2+}\) release. (Circ Res. 2007;101:000-000.)

Key Words: calcium-induced calcium release ■ excitation–contraction coupling

Cardiac myocyte excitation–contraction coupling (ECC) occurs by Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR),\(^{1,3}\) where a small Ca\(^{2+}\) current (ICa) through L-type Ca\(^{2+}\) channels (LCCs) locally controls a larger Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) via a closely apposed cluster of ryanodine receptors (RyRs).\(^{4,5}\) Whole-cell SR Ca\(^{2+}\) release magnitude is finely graded by the amplitude of ICa, and both variables have similar, but not identical, bell-shaped dependence on membrane voltage (V\(_m\)).\(^{6}\) However, these events have underlying unitary components with different V\(_m\) dependences. There are \(\approx\)30 000 spatially discrete junctions or dyads per myocyte, each of which contains an average of \(\approx\)12 LCCs and \(\approx\)100 RyRs,\(^{4,7}\) although the precise number of channels has variance that may be functionally important.\(^{8}\) Whole-cell Ca\(^{2+}\) transients result from the temporal and spatial summation of many independent Ca\(^{2+}\) release events known as Ca\(^{2+}\) sparks that are synchronized by ICa\(^{5,13}\) and the characteristics of which are V\(_m\) independent.\(^{11,14}\)

ICa is the ensemble of single channel currents (i\(_{Ca}\)) through thousands of individual open channels (NP\(_o\); ICa=i\(_{Ca}\)NP\(_o\)), and i\(_{Ca}\) amplitude and NP\(_o\) both depend on V\(_m\) (Figure 1d).\(^{15}\) At each dyad, CICR efficacy could be governed by either i\(_{Ca}\) amplitude or by local NP\(_o\), (which should parallel overall NP\(_o\)). Earlier voltage-clamp studies measuring global SR Ca\(^{2+}\) release or Ca\(^{2+}\) sparks\(^{11,13}\) in voltage steps from constant holding potential (V\(_{hold}\)) showed that the V\(_m\) dependence of ECC gain was similar to that predicted for i\(_{Ca}\). They inferred that higher i\(_{Ca}\) amplitude at negative V\(_m\) increases “coupling fidelity” (ie, the probability that i\(_{Ca}\) triggers an open channel triggers a Ca\(^{2+}\) spark), causing the increase in CICR gain. However, because NP\(_o\) also depends on V\(_m\) (over the same range), NP\(_o\) changes might also be important, and no prior study separated the impact of i\(_{Ca}\) versus NP\(_o\) on CICR gain. That is our aim here.

Measuring local SR Ca\(^{2+}\) release events during ECC is limited by spatial overlap of signals from neighboring junctions. Spatial separation of release events requires either drastic reduction of the number of active LCCs (by negative V\(_{test}\) or channel blockers)\(^{11,13}\) or trapping released Ca\(^{2+}\) by mM EGTA in combination with a fast local Ca\(^{2+}\) indicator (Ca\(^{2+}\) spikes).\(^{16,19}\) Here we measure Ca\(^{2+}\) spikes during voltage clamp to separate the role of i\(_{Ca}\) and NP\(_o\) in controlling...
ECC efficacy. This approach also ensures constant cytosolic and SR [Ca\(^{2+}\)] at each pulse. Surprisingly, we found that increasing either \(i_{c,a}\) or \(P_{a}\) alone decreases ECC gain. The results also suggest that the small \(i_{c,a}\) at a \(V_{T}\) of 0 mV may be a highly effective trigger of SR Ca\(^{2+}\) release and also that redundancy of Ca\(^{2+}\) channel opening at individual junctions is critical in how local control of SR Ca\(^{2+}\) release occurs.

**Materials and Methods**

**Voltage Clamp**

Enzymatically isolated rat ventricular myocytes were voltage clamped in a ruptured patch whole-cell configuration, allowing intracellular dialysis with a solution containing (in mmol/L): 115 CsCl, 10 NaCl, 10 tetraethylammonium chloride, 1 MgCl\(_2\), 5 MgATP, 0.3 NaGTP, 20 Hepes, 1 CaCl\(_2\), 10 glucose, 10 Hepes, and 0.02 tetrodotoxin, pH 7.4 with NaOH. Cells were dialyzed for 
faced with a confocal scan head (Radiance 2100, controlled by Lasersharp 2000 software; Bio-Rad) using a Plan Fluor \(\times 40\), 1.3 NA oil immersion objective lens. OG-5N was excited at 488 nm (argon laser at \(\approx 3\%\) to 6\% of maximum), with emission collected at 
>500 nm. Confocal data were acquired in line-scan mode at 500 Hz (with a pixel size of 120 nm and pinhole optimized for

**Confocal Imaging of Ca\(^{2+}\) Release Flux**

An inverted microscope (Eclipse, TE-2000-U, Nikon) was interfaced with a confocal scan head (Radiance 2100, controlled by Lasersharp 2000 software; Bio-Rad) using a Plan Fluor \(\times 40\), 1.3 NA oil immersion objective lens. OG-5N was excited at 488 nm (argon laser at \(\approx 3\%\) to 6\% of maximum), with emission collected at >500 nm. Confocal data were acquired in line-scan mode at 500 Hz (with a pixel size of 120 nm and pinhole optimized for resolution of \(\approx 0.4\) μm in the focal plane and <1 μm in the z-axis. Image processing used an algorithm written in IDL (Research Systems) provided by Dr H. Cheng (NIH, Baltimore, Md). Fluorescent images are normalized as F/F\(_0\), where F is fluorescence intensity and F\(_0\) is average fluorescence at rest. The fraction

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**Figure 1.** Voltage dependence of \(i_{c,a}\) and SR Ca\(^{2+}\) release in OG-5N/EGTA-loaded myocytes. a, Two-dimensional image and longitudinal scan-line position. Normalized \((F/F_0)\) line-scan image during depolarization from \(V_{T}=\)−60 to 0 mV (right); ordinate is distance and abscissa is time. Trace below is spatially averaged \(F/F_0\) (bar=0.1 \(\Delta F/F_0\)). The inset enlargement shows local Ca\(^{2+}\) release events. b, Peak \(i_{c,a}\) (filled) and whole-cell SR Ca\(^{2+}\) release flux (open) \((\Delta F/F_0)\) vs \(V_{T}\) (n=7 to 8). c, CICR gain (as peak \(\Delta F/F_0\) per peak \(i_{c,a}\) vs \(V_{T}\) at 140 μmol/L), which was constant throughout this protocol. Because tail currents can be contaminated equally, we repeated protocols in the same cell with CdCl\(_2\) (1 mmol/L) present, and used the Cd\(^{2+}\)-sensitive tail currents as \(i_{c,a}\).
of active junctions during voltage-clamp pulses (200 ms) was determined using a spike threshold of $\Delta F/F_0$ of 0.2. For comparing SR Ca$^{2+}$ release among different protocols, data were normalized to values for a depolarization from $V_{\text{hold}}$ of −60 to 0 mV with $[\text{Ca}^{2+}]_o = 1$ mmol/L. For the tail $I_{\text{Ca}}$ protocol, values were normalized to those on repolarization to 0 mV.

Statistics
The data are presented as means±SEM. Paired Student’s $t$ test or ANOVA, followed by all pairwise multiple comparison, were used when appropriate. $P<0.05$ was considered significant.

Results

Ca$^{2+}$-Induced Ca$^{2+}$ Release Gain, Membrane Potential, and Fractional Release
We measured whole-cell $I_{\text{Ca}}$ and individual local SR Ca$^{2+}$ release (Ca$^{2+}$ spikes) with confocal microscopy (Figure 1a). Ca$^{2+}$ spikes are discernible and proportional to release rate, as Ca$^{2+}$ transiently binds to OG-5N before being absorbed by EGTA. Electrically (and caffeine) evoked Ca$^{2+}$ spikes occur at regular intervals ($\approx 2\ \mu$m) along the length of the cell, corresponding to the location of transverse tubule–SR junctions at the z-line of each sarcomere. These represent junctional SR Ca$^{2+}$ release flux underlying ECC. The peak of the spatially averaged fluorescence ($\Delta F/F_0$) is an index of overall SR Ca$^{2+}$ release flux. Figure 1b displays the typical bell-shaped $V_m$ dependence of $I_{\text{Ca}}$, and the whole-cell SR Ca$^{2+}$ release flux ($\Delta F/F_0$). The classic $V_m$ dependence of ECC gain computed from these data are in Figure 1c. Because $I_{\text{Ca}}$ and NP$_o$ are $V_m$ sensitive (and change in opposite directions; Figure 1d), the rising phase of the gain curve, in principle, could be attributable to either increasing $I_{\text{Ca}}$, decreasing NP$_o$, or both. Therefore, to assess the independent roles of NP$_o$ and $I_{\text{Ca}}$ on CICR gain, we devised voltage-clamp protocols to isolate their impact, independent of $V_m$ at constant SR Ca$^{2+}$ load.

Fast caffeine-induced Ca$^{2+}$ release (Figure 2a) was used to verify the stability of SR Ca$^{2+}$ load. It also allowed us to identify all release sites along a scan line ($\approx 100\%$ active junctions) and to infer the overall fractional release of Ca$^{2+}$ from the SR (using the pedestal of $[\text{Ca}^{2+}]_o$ elevation after the peak release sensed by OG-5N equilibrated with EGTA; vertical blue arrows). Whereas $I_{\text{Ca}}$ could often activate $\approx 80\%$ of junctions, the integrated SR Ca$^{2+}$ release was only $\approx 50\%$ of that released by caffeine (Figure 2b).

Altering the NP$_o$ at Constant $I_{\text{Ca}}$
The effect of altered NP$_o$, alone on CICR gain was assessed by varying Ca$^{2+}$ channel availability by changing $V_{\text{hold}}$ with Ca$^{2+}$ release evoked by depolarizations to the same $V_{\text{hold}}$ (such that $I_{\text{Ca}}$ was always identical; Figure 3a). Indeed, changes of $V_{\text{hold}}$ over this range do not prolong single channel open time and did not significantly alter normalized $I_{\text{Ca}}$ time course when SR Ca$^{2+}$ release was blocked (data not shown). Thus, changes in $I_{\text{Ca}}$ in Figure 3 reflect changes in NP$_o$. This allowed variation of NP$_o$ over a wide range ($>10$-fold; $n=23$ to 24). The confocal line-scan images show that the number of Ca$^{2+}$ spikes (active junctions) increase at negative $V_{\text{hold}}$ (higher NP$_o$; Figure 3c), and this was also evident as increased global Ca$^{2+}$ spike amplitude and integrated SR Ca$^{2+}$ release (see Figure 1 in the online data supplement at http://circres.ahajournals.org). Indeed, gradation of global SR Ca$^{2+}$ release flux with NP$_o$ is largely caused by the number of junctions firing (see linear correlation in Figure 3b). There was also a minor change in mean Ca$^{2+}$ spike amplitude (20% to 25%), which correlates with and may be secondary to the larger fraction of active junctions (see the online data supplement). This may be attributable to recruitment of multiple junctions within the confocal-assessed volume. ECC gain (fraction of active junctions/peak $I_{\text{Ca}}$) decreased monotonically as a function of $V_{\text{hold}}$ (with increasing NP$_o$), indicating that CICR is more efficient when fewer LCCs open (Figure 3d). Thus reduced NP$_o$ could explain the increasing ECC gain at negative $V_m$ in Figure 1c. However, can increasing $I_{\text{Ca}}$ also explain it?

Altering $I_{\text{Ca}}$ at Constant NP$_o$
In the limiting case, where $I_{\text{Ca}}$ is nearly 0, it may not activate SR Ca$^{2+}$ release, but the intrinsic $I_{\text{Ca}}$ dependence of gain has not been previously measured. Here we altered $I_{\text{Ca}}$ amplitude (at constant NP$_o$) using 2 different, but complementary, protocols. First, $I_{\text{Ca}}$ was varied by abruptly changing $[\text{Ca}^{2+}]_o$ just before (and only during) the depolarization to a constant $V_{\text{ext}}$ (ensuring constant NP$_o$). Figure 4a shows $I_{\text{Ca}}$, confocal line scans, and spatially averaged Ca$^{2+}$ release in a representative cell. The fraction of active junctions decreased as $[\text{Ca}^{2+}]_o$ and $I_{\text{Ca}}$ were lowered, especially at $[\text{Ca}^{2+}]_o \leq 1$ mmol/L. These results show 2 key points. First, most of the drop in the fraction of junctions firing (Figure 4b) occurs only at very low $I_{\text{Ca}}$ ($[\text{Ca}^{2+}]_o < 0.5$ mmol/L) and is not increased as $I_{\text{Ca}}$ is elevated by raising $[\text{Ca}^{2+}]_o$ from 1 to 10 mmol/L. This implies...
that the small \( i_{\text{Ca}} \) at 0 mV has a high coupling fidelity and can effectively trigger \( \text{Ca}^{2+} \) release and that raising \( i_{\text{Ca}} \) does not evoke additional release. Second, increasing \( i_{\text{Ca}} \) over a broad range causes a monotonic decline in ECC gain (Figure 4c). Both of these points indicate that increasing \( i_{\text{Ca}} \) (eg, at more negative \( V_{m} \)) neither enhances coupling fidelity nor increases ECC gain (in contrast with earlier interpretations).

The second method to manipulate \( i_{\text{Ca}} \) at constant \( N_{\text{Po}} \) extended this analysis to lower \( i_{\text{Ca}} \) and also simulated better physiological action potentials (APs), where real ECC occurred. Here (Figure 5a), we first fully activated channels (high \( N_{\text{Po}} \)) by depolarization to +120 mV (which prevents \( \text{Ca}^{2+} \) entry). After 15 ms, the cell was repolarized to different \( V_{\text{test}} \) to increase \( \text{Ca}^{2+} \) driving force and \( i_{\text{Ca}} \) (tail current), but with the same initial \( N_{\text{Po}} \), at each pulse. The magnitude of \( \text{Ca}^{2+} \) release (and fraction of active junctions) was largest with a \( V_{\text{test}} \) near 0 mV (largest tail \( I_{\text{Ca}} \)) and decreased at more positive \( V_{\text{test}} \) (Figure 5a and 5b). Because LCCs were preactivated during the pulse to +120 mV, the mean \( \text{Ca}^{2+} \) spike latency was short and unaffected by \( V_{\text{test}} \) (supplemental Figure II). Figure 5c shows that ECC gain increases as \( i_{\text{Ca}} \) is reduced, even at the lowest \( i_{\text{Ca}} \). This is surprising, because \( i_{\text{Ca}} \) amplitude ought to be limiting at some level. However, these data suggest that under relatively physiological conditions, at positive \( V_{m} \), the intrinsic low coupling fidelity of small \( i_{\text{Ca}} \) may be counteracted by the synchronous activation of multiple LCCs.

**Composite Results**

Figure 6a illustrates the relative efficacy of \( I_{\text{Ca}} \) with different underlying unitary properties, in recruiting local SR \( \text{Ca}^{2+} \) release events. At an \( I_{\text{Ca}} \) of \( \pm 8 \) pA/pF, the experimental conditions are the same. However, as one lowers \( i_{\text{Ca}} \) by reducing \( N_{\text{Po}} \), the drop off in ECC efficacy is more severe when \( N_{\text{Po}} \) is reduced (red open and blue open, for the same \( i_{\text{Ca}} \) value). Thus a larger number of \( \text{Ca}^{2+} \) release events are recruited when \( i_{\text{Ca}} \) is small (but \( N_{\text{Po}} \) is high) than when \( N_{\text{Po}} \) is low (but \( i_{\text{Ca}} \) large). This underscores the importance of \( N_{\text{Po}} \) in regulating CICR efficacy.

**Discussion**

Cardiac SR \( \text{Ca}^{2+} \) release is graded by \( I_{\text{Ca}} \), but which unitary component (\( i_{\text{Ca}} \) versus \( N_{\text{Po}} \)) controls CICR gain at different \( V_{m} \).
is controversial. Because gain has similar $V_m$ dependence as the expected $i_{\text{Ca}}$ (Figure 1c and 1d), this was taken to indicate that the increase in $i_{\text{Ca}}$, amplitude enhances its capability to trigger release (higher coupling fidelity) and dictates high gain at negative $V_m$. However, the explicit $i_{\text{Ca}}$ dependence of gain or coupling fidelity is unknown. NP$_o$ is also influenced by $V_m$ over the same range as $i_{\text{Ca}}$ (Figure 1d), and no previous work has separated the influence of NP$_o$ and $i_{\text{Ca}}$, but rather both components change simultaneously as $V_m$ varies.$^{10-13}$

Our aims were to separately measure NP$_o$ and $i_{\text{Ca}}$ dependence of CICR and to better explain the $V_m$ dependence of ECC gain. Our data show that reducing either $i_{\text{Ca}}$, or NP$_o$ alone increased CICR gain and that a decrease in NP$_o$ has a stronger effect on the recruitment of Ca$^{2+}$ release sites than does a decrease in $i_{\text{Ca}}$. We suggest that the amplitude of $i_{\text{Ca}}$ at 0 mV (with 1 mmol/L [Ca$^{2+}$]) may be sufficient to effectively trigger uniform SR Ca$^{2+}$ release. Hence, additional $i_{\text{Ca}}$ (either as $i_{\text{Ca}}$ or NP$_o$) will be redundant or wasted, because it will not trigger additional Ca$^{2+}$ sparks (and thus decreases ECC gain). This disproves the idea that increasing $i_{\text{Ca}}$ at negative $V_m$ can increase gain. However, at positive $V_m$, where $i_{\text{Ca}}$ is rather small, effective SR Ca$^{2+}$ release might require synchronous activation of a group of LCCs. Moreover, the decline in SR Ca$^{2+}$ release that occurs at very positive square $V_m$ pulses must be mainly attributable to the progressively decline in $i_{\text{Ca}}$, because NP$_o$ is expected to remain maximal.

CICR Gain and Fractional Release

The OG-5N/EGTA fluorescence signals provide an accurate estimate of global and junctional SR Ca$^{2+}$ release.$^{16}$ Here we counted active junctional Ca$^{2+}$ release sites (Ca$^{2+}$ spikes) to assess CICR gradation. Notably, similar results were obtained when the whole-cell fluorescence peak was used to assess SR Ca$^{2+}$ release (see linear correlation in Figure 3b).

Because altered SR Ca$^{2+}$ content can dramatically affect ECC gain,$^{26}$ presumably because of an effect of intra-SR [Ca$^{2+}$] on RyR gating,$^{27}$ SR Ca$^{2+}$ content must be controlled in studies of CICR gain. Here we accomplished this by dialis of the cell with strongly buffered [Ca$^{2+}$], and long times between pulses (17 seconds). We demonstrated that despite the high concentration of these Ca$^{2+}$ chelators, total SR Ca$^{2+}$ release was consistent. Fractional SR Ca$^{2+}$ release on strong depolarization ($V_{\text{test}}=0$ mV) was $\approx 50\%$ of that evoked by caffeine, consistent with previous estimates without Ca$^{2+}$ chelators.$^{26}$ Thus, this method of trapping released Ca$^{2+}$ does not greatly modify the release process itself. This is convenient but may also mean that the shut-off of SR Ca$^{2+}$ release does not depend entirely on [Ca$^{2+}$] outside the SR (which should be limited somewhat by 1 mmol/L of the fast Ca$^{2+}$ buffer OG-5N). It would be consistent with some contribution of decreasing intra-SR Ca$^{2+}$ in the termination of SR Ca$^{2+}$ release.$^{28,29}$

Figure 1b shows that, with these Ca$^{2+}$ chelators, the classic $V_m$ dependence of CICR$^{10-13,17}$ is recapitulated. Previous reports with different experimental details all came to the same reasonable conclusion that increasing $i_{\text{Ca}}$ could explain why ECC gain rises at increasingly negative test $V_m$. However, none of them separated the impact of $i_{\text{Ca}}$ versus NP$_o$ as we have done to further test this conclusion.
NPo and CICR Gain

The actual number of LCCs at a single dyad varies, but an average dyad probably contains \( \sim 12 \) LCCs. Only a fraction of these open on any given pulse, and the average number, NPo, in a single dyad, should reflect global NPo. We found that reducing \( I_{\text{Ca}} \) by lowering NPo increases gain while reducing the number of active junctions (Figure 3c and 3d), presumably by limiting the number of Ca\(^{2+} \) channel openings at a given junction and the junctions where any openings happen. This may explain why, at more negative \( V_m \) (where NPo is low, but \( i_{\text{Ca}} \) is large), ECC gain is high because there is almost no redundant \( I_{\text{Ca}} \). That is, a single Ca\(^{2+} \) channel opening can trigger a Ca\(^{2+} \) spark at 1 dyad, and because P_o is very low, there are almost no dyads where more than 1 Ca\(^{2+} \) channel opens (which would be redundant or wasted openings). As \( V_m \) becomes less negative and P_o increases, multiple channels will open at some dyads; however, if the first opening suffices to trigger Ca\(^{2+} \) release, then subsequent openings are redundant for ECC. This will be true as long as the coupling fidelity of \( i_{\text{Ca}} \) at 0 mV is sufficient, and our data that increasing \( i_{\text{Ca}} \) always decreases gain suggest that this may be the case.

The latency of Ca\(^{2+} \) spike recruitment should be related to Ca\(^{2+} \) channel first opening latency. For high NPo (and adequate \( i_{\text{Ca}} \)), a first opening will occur earlier on average (even with opening being random). Accordingly, we found that the average latency of Ca\(^{2+} \) spikes was dramatically increased (\( \sim 2 \) fold) at low NPo (supplemental Figure II).

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**Figure 5.** \( i_{\text{Ca}} \) vs SR Ca\(^{2+} \) release flux evoked by repolarization. a, Voltage-clamp protocol and Cd\(^{2+} \)-sensitive tail \( I_{\text{Ca}} \) (constant [Ca\(^{2+} \)] \(_o\) = 1 mmol/L). At \( V_{\text{test}} = +50 \) mV, no Ca\(^{2+} \) was released until repolarization to \( V_{\text{hold}} \). b, Pooled peak \( I_{\text{Ca}} \) data (closed circles) and fraction of active junctions (open circles) as a function of \( V_{\text{test}} \) (n=8). Curve indicates single exponential fit to mean \( I_{\text{Ca}} \) (\( k=0.04 \)). c, CICR gain plotted vs trigger \( I_{\text{Ca}} \).

**Figure 6.** Composite results from various NPo and \( i_{\text{Ca}} \). a, Relative efficacy of NPo and \( i_{\text{Ca}} \) to activate local Ca\(^{2+} \) release events. Fraction of active junctions plotted vs trigger \( I_{\text{Ca}} \) with variable NPo (black closed circles) or \( i_{\text{Ca}} \) (open circles). b, CICR gain as a function of trigger \( I_{\text{Ca}} \).
Although 1 open LCC may suffice to trigger CICR at each site,\textsuperscript{12,13,19} a cluster of functionally available LCCs might ensure that at least 1 will open on depolarization and will help trigger relatively synchronized Ca\textsuperscript{2+} release.\textsuperscript{19,30} Although multiple openings create a safety margin to assure high fidelity synchronized signaling,\textsuperscript{19} this also creates redundant (or wasted) Ca\textsuperscript{2+} entry and reduction in gain at higher \(i_{Ca}\) (high NP\(_o\)).

The precise number of active LCCs necessary to trigger a Ca\textsuperscript{2+} spark at all \(V_m\) is not known, but the following simple numbers may provide quantitative perspective. An \(i_{Ca}\) of 8 pA/pF during the first 20 ms in a 30-\mu L myocyte would require \(\approx 90,000\) single channel openings (for \(i_{Ca}\) of 0.33 pA for 0.5 ms) or \(\approx 25\%\) of the Ca\textsuperscript{2+} channels. If there are \(\approx 30,000\) junctions per myocyte, this implies an average of \(\approx 3\) LCC openings per junction. It is thus not surprising that reducing NP\(_o\) rapidly reduces the fraction of active junctions from the relatively large value at \(V_m=0\) mV (Figure 6a). We did not study the \(\approx 30\%\) of LCCs on the surface sarcolumns,\textsuperscript{31} but these likely also colocalize with RyRs at surface junctions.\textsuperscript{8} Although Brette et al.\textsuperscript{12,23} measured slower \(i_{Ca}\) inactivation for surface versus transverse tubular \(i_{Ca}\), they reported similar ECC gain for both.

### Single L-Type Ca\textsuperscript{2+} Channel Current Amplitude and CICR Gain

We tested whether increasing \(i_{Ca}\) amplitude increases gain and coupling fidelity (using rapid [Ca\textsuperscript{2+}] changes at constant NP\(_o\)). SR Ca\textsuperscript{2+} release and percentage of active junctions were nearly maximum at [Ca\textsuperscript{2+}]\(_i=1\) mmol/L, with little increase at 10 mmol/L (despite nearly double the \(i_{Ca}\); Figure 4b). This indicates that the coupling fidelity for \(i_{Ca}\) at [Ca\textsuperscript{2+}]\(_i=1\) mmol/L (at 0 mV) is near optimal for CICR. Extra Ca\textsuperscript{2+} influx then increases the denominator in the gain equation without commensurate SR Ca\textsuperscript{2+} release enhancement, such that gain decreases at higher \(i_{Ca}\). Similar decreases in ECC gain are seen with long channel openings induced by Bay K 8644,\textsuperscript{34} where presumably the initial \(i_{Ca}\) is sufficient to trigger release (and the rest is redundant).

We also used a voltage clamp protocol more like an action potential (Figure 5), where the range of \(i_{Ca}\) analysis was extended to smaller values than in Figure 4. This protocol with a brief prepulse to +120 mV mimics the AP upstroke in rapidly activating a large number of Ca\textsuperscript{2+} channels but without Ca\textsuperscript{2+} influx (constant NP\(_o\)). Then sudden repolarization increases the Ca\textsuperscript{2+} electrochemical gradient, causing a rapid influx through open channels, as occurs during early AP repolarization.\textsuperscript{18} Still ECC gain increased at even smaller \(i_{Ca}\) (Figure 5c). This is surprising because, at some very low \(i_{Ca}\), one should see ineffective triggering of Ca\textsuperscript{2+} release (low coupling fidelity) and hence decreased gain. Notably, the results for pseudo-AP pulses (Figure 5b) differ from usual square pulses to positive \(V_m\) (Figure 1b). With the positive prepulse SR, Ca\textsuperscript{2+} release declines less dramatically with decreasing \(i_{Ca}\) than for the traditional square pulse. That is, it takes a 6-fold decrease in \(i_{Ca}\) in the +prepulse experiment (Figure 5b) to reduce SR Ca\textsuperscript{2+} release by 50%, whereas only a 3-fold \(i_{Ca}\) reduction in the classic square pulse experiment (Figure 1b) produces a comparable decrease in SR Ca\textsuperscript{2+} release. We pose 2 explanations.

First, high NP\(_o\) with strong depolarization (or AP peak) may synchronize tail \(i_{Ca}\) (or peak \(i_{Ca}\) during early AP repolarization) even when \(i_{Ca}\) amplitude is small, thereby enhancing coupling fidelity (eg, simultaneous subthreshold \(i_{Ca}\) summate to assure triggering). Square pulses to lower \(V_m\) may activate many channels, but less synchronously, so SR Ca\textsuperscript{2+} release may be less reliably triggered. This is consistent with Sah et al.\textsuperscript{18} who showed that Ca\textsuperscript{2+} spark frequency during an AP depends strongly on early repolarization rate, and we found shorter latency of Ca\textsuperscript{2+} spikes during the tail \(i_{Ca}\) protocol, regardless of \(V_m\) (supplemental Figure II). Hence, during APs, the expected decrease in \(i_{Ca}\) coupling fidelity may be counteracted by the well-synchronized high number of open channels.

Second, the strong positive prepulse or AP peak may drive Ca\textsuperscript{2+} influx via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (before Ca\textsuperscript{2+} entry occurs via LCCs).\textsuperscript{12,35–40} Whereas some data with action potential and voltage-clamp–induced Ca\textsuperscript{2+} transients\textsuperscript{41,42} suggest that Ca\textsuperscript{2+} entry via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange is not critical for physiological ECC, our present results leave open the following possibility. This early Ca\textsuperscript{2+} entry may not trigger Ca\textsuperscript{2+} release by itself but could elevate local [Ca\textsuperscript{2+}], such that a smaller, and possibly well-synchronized, local \(i_{Ca}\) exhibits higher coupling fidelity (again, versus the simple square pulse).

In conclusion, our results suggest that the relatively small \(i_{Ca}\) at 0 mV (with 1 mmol/L [Ca\textsuperscript{2+}]\(_i\)) is sufficient for triggering Ca\textsuperscript{2+} sparks, such that a single channel opening may trigger SR Ca\textsuperscript{2+} release (even at 0 mV). At more negative \(V_m\), the gain is increased because there is less redundancy of LCC openings per junction (lower NP\(_o\), and this effect more than offsets the increasing \(i_{Ca}\) (which by itself decreases gain). At positive \(V_m\) (with square depolarizations), where \(i_{Ca}\) becomes much smaller, the decline in SR Ca\textsuperscript{2+} release and ECC gain may be related to a decrease in \(i_{Ca}\) coupling fidelity, despite multiple channel openings. Finally, more efficient SR Ca\textsuperscript{2+} release during an AP may be achieved by synchronized opening of LCCs during the AP upstroke or bolstering of local [Ca\textsuperscript{2+}]\(_i\).


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Voltage Dependence of Cardiac Excitation-Contraction Coupling Unitary Ca$^{2+}$ Current Amplitude and Open Channel Probability
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Supplemental Figure I shows the influence of holding potential ($V_{\text{hold}}$) on Ca$^{2+}$ spike peak, integrated SR Ca$^{2+}$ release flux and the percent of junctions activated for the results in manuscript Figure 3. With reduced $I_{\text{Ca}}$ availability (more positive $V_{\text{hold}}$) the amplitude of SR Ca$^{2+}$ release flux progressively declines, as does the total amount released assessed from the pedestal of OG-5N signal (Suppl. Fig. 1b). This also parallels the decrease in the number of active junctions (Fig 3c). As the number of active junctions declines, there is also a reduction in Ca$^{2+}$ spike amplitude, but to a much smaller extent. The small decrease in spike amplitude, we think is due to the drop-out of Ca$^{2+}$ release events in nearby junctions above or below the plane of confocality. Thus, 80% of the maximal Ca$^{2+}$ spike amplitude probably represents the signal from a visible Ca$^{2+}$ spike, while the extra 20% may be due to contaminating signal from similar size events above and below (which is quantitatively compatible with the data and optical system).

Supplemental Figure II shows Ca$^{2+}$ spike latency for experiments where $I_{\text{Ca}}$ was reduced by lowering NPo as in Fig 3 (a), $i_{\text{Ca}}$ as in Fig 4 (b) or $i_{\text{Ca}}$ by pre-activating Ca$^{2+}$ channels at +120 mV as in Fig 5 (c). These latency values are similar to those reported by Song et al. with this approach. Note that reducing NPo (at constant $I_{\text{Ca}}$) results in increasing latency, which probably reflects the smaller number of Ca$^{2+}$ channels which can be activated at a given junction. That is, increasing the number of activated channels, enhances the likelihood that one will open early. In contrast, altering $i_{\text{Ca}}$ (at constant NPo) did not change spike latency (Suppl. Fig. IIb-c). However, latency was shorter for all $i_{\text{Ca}}$ when channels were pre-activated by depolarization to positive $V_m$ (tail $I_{\text{Ca}}$).

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