Sarcoplasmic Reticulum Ca\textsuperscript{2+} Release Causes Myocyte Depolarization

Underlying Mechanism and Threshold for Triggered Action Potentials

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Abstract—Spontaneous sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release causes delayed afterdepolarizations (DADs) via Ca\textsuperscript{2+}-induced transient inward currents ($I_{\text{Na}}$). However, no quantitative data exists regarding (1) Ca\textsuperscript{2+} dependence of DADs, (2) Ca\textsuperscript{2+} required to depolarize the cell to threshold and trigger an action potential (AP), or (3) relative contributions of Ca\textsuperscript{2+}-activated currents to DADs. To address these points, we evoked SR Ca\textsuperscript{2+} release by rapid application of caffeine in indo 1-AM–loaded rabbit ventricular myocytes and measured caffeine-induced DADs (cDADs) with whole-cell current clamp. The SR Ca\textsuperscript{2+} load of the myocyte was varied by different AP frequencies. The cDAD amplitude doubled for every 88±8 nmol/L of $\Delta$[Ca\textsuperscript{2+}], (simple exponential), and the $\Delta$[Ca\textsuperscript{2+}] threshold of 424±58 nmol/L was sufficient to trigger an AP. Blocking Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange current ($I_{\text{NaCa}}$) by removal of [Na\textsubscript{i}] and [Ca\textsubscript{i}], (or with 5 mmol/L Ni\textsuperscript{2+}) reduced cDADs by >90%, for the same $\Delta$[Ca\textsuperscript{2+}]. In contrast, blockade of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current ($I_{\text{ClCa}}$) with 50 μmol/L niflumate did not significantly alter cDADs. We conclude that DADs are almost entirely due to $I_{\text{NaCa}}$, not $I_{\text{ClCa}}$ or Ca\textsuperscript{2+}-activated nonselective cation current. To trigger an AP requires 30 to 40 μmol/L cytosolic Ca\textsuperscript{2+} or a [Ca\textsuperscript{2+}], transient of 424 nmol/L. Current injection, simulating $I_{\text{Na}}$ with different time courses, revealed that faster $I_{\text{Na}}$ require less charge for AP triggering. Given that spontaneous SR Ca\textsuperscript{2+} release occurs in waves, which are slower than cDADs or fast $I_{\text{Na}}$, the true $\Delta$[Ca\textsuperscript{2+}] threshold for AP activation may be ~3-fold higher in normal myocytes. This provides a safety margin against arrhythmia in normal ventricular myocytes. (Circ Res. 2000; 87:774–780.)

Key Words: delayed afterdepolarization ■ sarcoplasmic reticulum ■ transient inward current ■ Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger ■ arrhythmia

A rrhythmias are a major cause of sudden cardiac death in heart failure (HF). Three-dimensional mapping indicates that nearly all ventricular tachycardias in nonischemic HF and ~50% of those in ischemic HF are initiated by nonreentrant mechanisms.\textsuperscript{1} These can arise from abnormal ventricular automaticity or triggered activity. The latter consists of either early afterdepolarizations (EADs) occurring in the plateau phase of the action potential (AP) or delayed afterdepolarizations (DADs), occurring at repolarized membrane potentials ($E_{\text{m}}$). Some EADs may be attributable to reactivation of Ca\textsuperscript{2+} channels, which can partially recover during long APs, especially as [Ca\textsuperscript{2+}], declines.\textsuperscript{3–6}

DADs, the focus in the present study, are generally thought to be initiated by spontaneous Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) and a Ca\textsuperscript{2+}-activated transient, depolarizing inward current ($I_{\text{Na}}$).\textsuperscript{7} Three candidates for $I_{\text{Na}}$ are Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange current ($I_{\text{NaCa}}$), Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current ($I_{\text{ClCa}}$), and Ca\textsuperscript{2+}-activated nonselective cation current ($I_{\text{NSCa}}$).\textsuperscript{8–10} Although there was early evidence implicating $I_{\text{NSCa}}$ as underlying $I_{\text{Na}}$,\textsuperscript{8} more recent work has not supported a major role for $I_{\text{NSCa}}$ in $I_{\text{Na}}$ or DADs of ventricular myocytes, favoring instead key roles for $I_{\text{ClCa}}$ and $I_{\text{NaCa}}$.\textsuperscript{6,9,11–14} In canine ventricular myocytes, Zygmunt et al\textsuperscript{2} attributed 60% of $I_{\text{Na}}$ to $I_{\text{NaCa}}$ and 40% to $I_{\text{ClCa}}$. The contributions of aforementioned currents to DAD generation may differ from those during an $I_{\text{Na}}$ (where $E_{\text{m}}$ is constant), because $E_{\text{m}}$ changes dynamically to alter the electrochemical driving force (most notably for $I_{\text{ClCa}}$) during DADs. One goal of the present study was to measure the relative contribution of $I_{\text{NSCa}}$, $I_{\text{NaCa}}$, and $I_{\text{NaCa}}$ to Ca\textsuperscript{2+}-activated depolarizations, leading to triggered APs.

Increasing SR Ca\textsuperscript{2+} load increases spontaneous SR Ca\textsuperscript{2+} release\textsuperscript{15} and DAD amplitude toward threshold to trigger an AP, the precursor of triggered arrhythmias.\textsuperscript{16} Although DADs are generally accepted to be Ca\textsuperscript{2+}-dependent,\textsuperscript{12,17} the relationship between SR Ca\textsuperscript{2+} release and DAD amplitude has not been measured, partly because the underlying Ca\textsuperscript{2+} transients are hard to control.

In the present study, we used caffeine-induced SR Ca\textsuperscript{2+} release to simulate DADs with different [Ca\textsuperscript{2+}], in a much more controlled manner and measured the resulting depolar-
zation. These caffeine-induced DADs (cDADs) can be initiated at various SR Ca\(^{2+}\) loads (eg, by changing frequency), allowing us to measure the [Ca\(^{2+}\)]\(_{\text{free}}\) dependence of DADs and triggered APs over a broad range of [Ca\(^{2+}\)]\(_{\text{free}}\). Spontaneous SR Ca\(^{2+}\) release typically occurs in waves,\(^{17}\) less synchronized than during application of caffeine or excitation-contraction coupling. Therefore, we injected \(I_\text{ls}\)-like current and measured membrane depolarization (\(\Delta E_m\)). These pseudo-\(I_\text{ls}\) mimic real \(I_\text{ls}\) but are Ca\(^{2+}\)-independent. The resulting \(\Delta E_m\) depends on other membrane properties such as \(I_{K_\text{atp}}\), the major background current that stabilizes resting \(E_m\). \(I_{K_\text{atp}}\) is reduced in HF,\(^{18,19}\) where arrhythmias are common.

The goals of the present study are to measure, for the first time, (1) the quantitative relationship between the amount of SR Ca\(^{2+}\) release and the amplitude of cDADs, (2) the amount of SR Ca\(^{2+}\) release (and \(E_m\)) required to reach threshold for an AP, (3) the specific contributions of different Ca\(^{2+}\)-activated currents that cause cDADs, and (4) the effect of different \(I_\text{ls}\) kinetics on \(\Delta E_m\) and AP threshold in the absence of [Ca\(^{2+}\)]\(_{\text{free}}\), changes. The results provide the first quantitative data on the basis of DADs and triggered APs in rabbit ventricular myocytes at 37°C.

**Materials and Methods**

Single ventricular myocytes from New Zealand White rabbits were isolated as previously described\(^{20}\) after injection of pentobarbital (50 mg/kg) and heparin. Rabbits were obtained from Myrtle’s Rabbitties (Thompson Station, Tenn) and cared for according to AAALAC guidelines. Myocytes were stored at 22°C and plated later for experiments on laminin pretreated glass-bottomed chambers on the stage of an inverted microscope equipped for epifluorescence.\(^{21}\)

Cells were loaded at 22°C with indo 1 (acetoxymethylester, 30-minute exposure, 30-minute washout). Indo 1 was excited at 365±25 nm and emitted fluorescence was measured at 405±10 and 485±10 nm. The fluorescence ratio \((R = F_{485}/F_{405})\) was translated as [Ca\(^{2+}\)]\(_{\text{free}}\) with \(K_\text{d} = 365\,\text{nm} / 405\,\text{nm}\) and \(K_\text{b} = 365\,\text{nm} / F_{485}\) (where \(K_\text{b}\) is the ratio of maximum to minimum \(F_{485}\) (4.4) and \(K_\text{b} = 365\,\text{nm} / 405\,\text{nm}\)). \(R_{\text{max}}\) is R at [Ca\(^{2+}\)]\(_{\text{free}}\) = <\(K_\text{b}\) and \(R_{\text{min}}\) is R at saturating [Ca\(^{2+}\)]\(_{\text{free}}\) (0.768 and 8.45 respectively; in vivo).

Whole-cell, ruptured-patch current clamp was used to measure \(E_m\) in response to caffeine and current injection. Electrodes (borosilicate) had resistance of 1 to 20 MΩ when filled with (mmol/L) potassium aspartate 120, KCl 8, NaCl 7, HEPES 10, MgCl\(_2\) 1, Mg-ATP 5, Li-GTP 0.3, K\(_5\)-indo 1 0.05 (included to prevent indo 1 block with Li\(_5\) as previously described\(^{20}\) after injection of pentobarbital (50 mg/kg) and heparin). Cells were loaded at 22°C with indo 1 (acetoxymethylester, 30-minute exposure, 30-minute washout). Indo 1 was excited at 365±25 nm and emitted fluorescence was measured at 405±10 and 485±10 nm. The fluorescence ratio \((R = F_{485}/F_{405})\) was translated as [Ca\(^{2+}\)]\(_{\text{free}}\) with \(K_\text{d} = 365\,\text{nm} / 405\,\text{nm}\) and \(K_\text{b} = 365\,\text{nm} / 405\,\text{nm}\). Cells were held at 37°C.

Normal Tyrode’s (NT) superfusate contained (mmol/L) NaCl 140, KCl 4, glucose 10, HEPES 5, MgCl\(_2\) 2, CaCl\(_2\) 2, pH 7.4 adjusted with NaOH at 37°C. Caffeine (10 mmol/L in NT) was applied by fast solution switching to release SR Ca\(^{2+}\) load. The SR Ca\(^{2+}\) load was varied either by AP frequency, rest interval, or partial reloading after a preceding caffeine application. Similar SR Ca\(^{2+}\) loads were attained for comparisons in the presence and absence of 50 µmol/L niflumate (to block \(I_{\text{NaCa}}\), 5 mmol/L Ni\(^{2+}\), or Na\(^+\)/Ca\(^{2+}\) solution (replacing Na\(^+\) with Li\(^+\) and Ca\(^{2+}\) with Mg\(^{2+}\) in addition to 10 mmol/L EGTA to block \(I_{\text{NaCa}}\)). These agents were applied rapidly for ~2 seconds before (and during) caffeine application.

For testing \(I_\text{ls}\) effects on \(E_m\) (independent of [Ca\(^{2+}\)]\(_{\text{free}}\)) in rabbit myocytes (\(I_{\text{ls}}(\text{basal})\)). The injected artificial \(I_\text{ls}\) was varied in amplitude (and kinetics) to define a cell-dependent threshold for triggering an AP. Data are shown as mean±SE, and statistical significance was considered for values of \(P<0.05\) (Student’s \(t\) test or ANOVA).

**Results**

**Dependence of Depolarization on SR Ca\(^{2+}\) Release**

SR Ca\(^{2+}\) load available for release was varied by changing AP frequency. Figure 1 shows the last steady-state (SS) AP and [Ca\(^{2+}\)]\(_{\text{free}}\) transient. After ~2 seconds, caffeine-induced [Ca\(^{2+}\)]\(_{\text{free}}\) transients generate subthreshold cDADs (1 to 3 Hz) and a suprathreshold cDAD/AP at 4 Hz. Inset shows that depolarization rises in phase with increasing [Ca\(^{2+}\)]. SS indicates steady state.

**Figure 1.** \(E_m\) and [Ca\(^{2+}\)]\(_{\text{free}}\) in rabbit ventricular myocytes at 37°C. Graph on the left shows the last SS AP and [Ca\(^{2+}\)]\(_{\text{free}}\) transient. With increasing frequency, the amplitude of the caffeine-induced Ca\(^{2+}\) transients and cDADs (right). With increasing frequency, the amplitude of the caffeine-induced Ca\(^{2+}\) transient increased, consistent with the expected increase in SR Ca\(^{2+}\) load. For 1- to 3-Hz stimulation, the cDADs remained subthreshold, but \(E_m\) changed as [Ca\(^{2+}\)]\(_{\text{free}}\) increased (Figure 1, inset). At 4 Hz, depolarization was sufficient to trigger a regenerative AP with a long, low plateau. The [Ca\(^{2+}\)]\(_{\text{free}}\) decline during this long AP was greatly slowed, presumably attributable to the decrease in driving force for inward \(I_{\text{NaCa}}\), the major sarcoplasmic Ca\(^{2+}\)-removal process.\(^{24}\)

Figure 2A shows the mean effect of frequency on cDAD and \(\Delta[\text{Ca}^{2+}]\), (subthreshold events only). Ca\(^{2+}\) transients increased progressively with frequency, and cDAD amplitude also rose with frequency from 6.6±0.8 mV at 0.5 Hz to 14.4±1.2 mV at 3 Hz. In contrast, there was no frequency-dependent change in diastolic [Ca\(^{2+}\)]\(_{\text{free}}\) (range: 154±16 mmol/L at 0.5 Hz to 169±16 mmol/L at 2 Hz; \(P=0.873\), ANOVA) or resting \(E_m\) (range: −76.4±0.6 mV at 1 Hz to −77.1±1.1 mV at 3 Hz; \(P=0.934\), ANOVA).

A wider range of subthreshold Ca\(^{2+}\) transients and cDADs were obtained by varying SR Ca\(^{2+}\) load by frequency and/or number of conditioning APs. Cells with four or more subthreshold [Ca\(^{2+}\)]-cDAD value pairs were fit to a simple-
exponential equation (Figure 2B). In 20 cells, the average \( \Delta [\text{Ca}^{2+}] \), causing a doubling of \( \Delta E_m \), was 88±8 mmol/L [Ca\(^{2+}\)], for subthreshold values.

In 12 cells, APs were triggered at higher \( \Delta [\text{Ca}^{2+}] \) values. Threshold was defined as the highest \( \Delta [\text{Ca}^{2+}] \) that failed to trigger an AP (424±58 mmol/L \( \Delta [\text{Ca}^{2+}] \)), at \( \Delta E_m = 12.5±1.1 \) mV, Figure 2B; peak \( [\text{Ca}^{2+}] \) was 608±72 mmol/L and \( E_m = -64.7±1.2 \) mV. The first \( \Delta [\text{Ca}^{2+}] \), to trigger an AP exceeded the threshold by 34±10 mmol/L [Ca\(^{2+}\)]. Thus, the true threshold \( \Delta [\text{Ca}^{2+}] \), may be slightly above our threshold, but within \(~34\) mmol/L.

Separation of Inward Currents Contributing to the Generation of DAD

Three different Ca\(^{2+}\)-activated currents (\( I_{\text{NS(Ca)}} \), \( I_{\text{NS(Ca)}} \), \( I_{\text{NS(Ca)}} \)) could contribute to DADs. Using AP trains that produced comparable caffeine-induced \( \Delta [\text{Ca}^{2+}] \), we evaluated the effect of abrupt block of these different currents on the amplitude of subthreshold cDADs. Figure 3A shows that elimination of \( I_{\text{NS(Ca)}} \) by removal of both extracellular substrates (2 seconds in 0Na/0Ca solution) nearly abolished cDADs. Because [Cl\(^{-}\)] was unchanged, \( I_{\text{NS(Ca)}} \) should be unaffected, and the replacement of Na\(^{+}\) with Li\(^{+}\) also ensures that \( I_{\text{NS(Ca)}} \) is fully functional. The half-time of [Ca\(^{2+}\)], decline during caffeine exposure is dramatically slowed in 0Na/0Ca solution (from 120 ms to 1.7 seconds in Figure 3A). This is consistent with \( I_{\text{NS(Ca)}} \) being responsible for >90% of [Ca\(^{2+}\)], decline in caffeine. Moreover, the slower [Ca\(^{2+}\)], decline ought to enhance other Ca\(^{2+}\)-activated currents (including \( I_{\text{NS(Ca)}} \)), but none was evident. Thus, cDADs seem to depend on \( I_{\text{NS(Ca)}} \) and not \( I_{\text{NS(Ca)}} \) or \( I_{\text{NS(Ca)}} \).

Ni\(^{2+}\) (5 mmol/L) is also commonly used to inhibit \( I_{\text{NS(Ca)}} \), although it affects Ca\(^{2+}\) and some other currents (but not \( I_{\text{NS(Ca)}} \)). Ni\(^{2+}\) superfusion for 2 seconds before and during caffeine application also blocked cDADs almost completely (Figure 3B) for a similar \( \Delta [\text{Ca}^{2+}] \). With 5 mmol/L Ni\(^{2+}\), the [Ca\(^{2+}\)], decline is slowed, but to a lesser extent (≈3-fold versus >10-fold for 0Na/0Ca solution). This indicates less complete block of \( I_{\text{NS(Ca)}} \) by 5 mmol/L Ni\(^{2+}\) than by 0Na/0Ca solution.

Figure 4A shows that blockade of \( I_{\text{Cl(Ca)}} \) by inclusion of 50 μmol/L niflumate before and during caffeine application had only a very small depressant effect on subthreshold cDADs (whereas \( \Delta [\text{Ca}^{2+}] \), induced by caffeine was virtually identical in both cases). The Cl\(^{-}\) reversal potential (\( E_{\text{Cl}} \)) under our experimental conditions (and physiologically) is about ~58 mV. Therefore, increased Cl\(^{-}\) conductance at resting \( E_m \) would lead to an inward current. However, as depolarization proceeds toward \( E_{\text{Cl}} \) (as in a DAD), the driving force for Cl\(^{-}\) will decrease considerably. This and the outward rectification of \( I_{\text{Cl(Ca)}} \) may explain why \( I_{\text{Cl(Ca)}} \) appears to contribute so little

![Figure 2](http://circres.ahajournals.org/)

*Figure 2. A, Subthreshold cDAD amplitude (\( \Delta E_m \)) and \( \Delta [\text{Ca}^{2+}] \) depend on SS stimulation frequency. \( \Delta E_m \) rises with frequency from 6.6±0.8 to 14.4±1.2 mV and \( \Delta [\text{Ca}^{2+}] \) from 316±28 to 479±73 mmol/L (P<0.05 vs 0.5 Hz). B, Exponential \( \Delta [\text{Ca}^{2+}] \) dependence of subthreshold cDADs [\( \Delta E_m = 0.4 \) mV-exp(K·\( \Delta [\text{Ca}^{2+}] \)]. Fits for 7 of 20 cells are shown. Mean fit (bold curve) doubles \( \Delta E_m \) in [2/K] for every 88±8 mmol/L [Ca\(^{2+}\)]. (range: 21 to 156 mmol/L [Ca\(^{2+}\)]). Threshold [Ca\(^{2+}\)] to trigger an AP was 424±58 mmol/L with \( \Delta E_m \) of 12.5±1.1 mV (n=12).*

![Figure 3](http://circres.ahajournals.org/)

*Figure 3. cDAD before and after \( I_{\text{NS(Ca)}} \) blockade by 0Na/0Ca (A) or 5 mmol/L Ni\(^{2+}\) (B) for 2 seconds. Traces were selected to match \( \Delta [\text{Ca}^{2+}] \) in a given cell before and after block of \( I_{\text{NS(Ca)}} \) (0.5 to 1 Hz SS). Blockade of \( I_{\text{NS(Ca)}} \) inhibited cDADs and [Ca\(^{2+}\)], decline in the presence of caffeine.*
to the cDAD, despite the presence of this current in rabbit ventricular myocytes at 37°C.27,28

$\text{I}_{\text{Cl(Ca)}}$ is thought to contribute to early repolarization of the ventricular AP. Figure 4A (inset) shows that 50 μmol/L niflumate (as used in the present study) inhibits early repolarization seen as diminished notch in the AP. Niflumate also increased AP duration. These data provide an internal positive control for niflumate effects on $\text{I}_{\text{Cl(Ca)}}$.

Figure 4B summarizes the effect of 0Na/0Ca, Ni$^{2+}$, and niflumate on subthreshold cDADs. 0Na/0Ca solution and Ni$^{2+}$ almost completely inhibited cDADs (by 93% and 91%, respectively) for comparable $\Delta[\text{Ca}^{2+}]$ (bottom; n=12, 7, and 5; paired).

Figure 5 shows that when a cDAD was sufficient to trigger an AP, the AP could be completely blocked by 0Na/0Ca solution (where Li$^+$ can carry Na$^+$ channel current). However, niflumate did not prevent the triggered AP (although it decreased early repolarization). This confirms that the key current for SR Ca$^{2+}$ release–triggered APs is $I_{\text{NaCa}}$, not $I_{\text{Cl(Ca)}}$ or $I_{\text{NS(Ca)}}$.

Membrane Response to Current Injection at Resting Membrane Potential

To test the Ca$^{2+}$-independent effects of $I_q$ kinetics on membrane depolarization in a controlled manner, we injected inward currents mimicking Ca$^{2+}$-activated $I_q$ of different amplitudes and kinetics. We used three scalable current injection templates (see Figure 6A). The slowest ($I_{q,\text{slow}}$) resembles a measured $I_q$, which is produced by a spontaneous [Ca$^{2+}$]i wave traveling through the cell. The faster time courses ($I_{q,\text{mid}}$; $I_{q,\text{fast}}$) resemble the kinetics of $I_q$s with greater synchronization of SR Ca$^{2+}$ release (as induced by rapid caffeine application). These pseudo-$I_q$s allowed us to simulate, in a controlled manner, the depolarizing impact expected...
Exponential fits yielded a doubling of $D_{t_i,fast}$ to 1.69 C/F for $I_{t_i,fast}$ (n = 5), whereas the $I_{t_i,slow}$ required 0.416 C/F to double $D_{E_m}$ (n = 17). In Table, $I_{t_i,slow}$ required 3 times as much charge as $I_{t_i,fast}$ for a given $D_{E_m}$. The average charge necessary to trigger an AP also increased progressively from 0.47 C/F for $I_{t_i,fast}$ (n = 7), whereas the $I_{t_i,slow}$ required 0.416 C/F to double $D_{E_m}$ (n = 17, see Table). In addition, $I_{t_i,slow}$ required $\approx 3$ times as much charge as $I_{t_i,fast}$ for a given $D_{E_m}$. The average charge necessary to trigger an AP also increased progressively from 0.47 C/F for $I_{t_i,fast}$ to 1.69 C/F for $I_{t_i,slow}$ (Table). Threshold $E_m$ was slightly, but not more significantly, positive in the case of $I_{t_i,slow}$ versus $I_{t_i,fast}$ ($-59 \pm 1$ versus $-63 \pm 3$ mV).

The integrated $I_{t_i,slow}$ can be converted to an equivalent $Ca^{2+}$ flux via $I_{Ca^{2+}}$. That is, 0.89 C/F corresponds to $I_{Ca^{2+}}$, Ca$^{2+}$ extrusion of 59.6 μmol/L cytosol (assuming a surface-to-volume ratio of 6.44 pF/pL cytosol), requiring SR Ca$^{2+}$ release of $\approx 64$ μmol/L cytosol (assuming 93% of released Ca$^{2+}$ is extruded by $I_{Ca^{2+}}$). Taking cytosolic Ca$^{2+}$ buffering into account, $[Ca^{2+}]_i$ would be raised by $\approx 428$ nmol/L. This is in remarkable agreement with the $\Delta [Ca^{2+}]_i$ threshold for triggering an AP via cDAD, on the basis of data in Figure 2B (424 nmol/L).

**Mechanism Underlying DADs: Ca$^{2+}$-Activated Currents**

Several Ca$^{2+}$-activated currents have been proposed to participate in $I_m$ and DADs, namely $I_{Na/Ca}$, $I_{Cl(Ca)}$, and $I_{NS(Ca)}$. Our data clearly indicate that in rabbit ventricular myocytes at 37°C, DADs are almost entirely attributable to $I_{Na/Ca}$ ($>90\%$) with $<10\%$ attributable to $I_{Cl(Ca)}$ and no evidence for contribution from $I_{NS(Ca)}$.

Many $I_m$ studies use voltage clamp, where $E_m$ is held constant. This has an inherent mechanistic bias and may not properly estimate relative current contributions to DADs (as measured in the present study). For example, with $E_m = -58$ mV under physiological conditions, an $E_m$ change from $-78$ to $-68$ mV during a DAD would reduce the driving force for $I_{Ca^{2+}}$ by 50%, leading to overestimation of the $I_{Ca^{2+}}$ contribution in a voltage-clamp experiment. In contrast, $E_{Na/Ca}$ is $\approx -30$ mV at rest in rabbit, but as $[Ca^{2+}]_i$ rises, $E_{Na/Ca}$ becomes more positive (eg, $+10$ mV) greatly enhancing the driving force for inward $I_{Na/Ca}$, more than offsetting the $D_{E_m}$-induced reduction of $I_{Na/Ca}$ driving force. Allosteric activation of $I_{Ca^{2+}}$ by $[Ca^{2+}]_i$ could further stimulate inward $I_{Na/Ca}$.

In voltage-clamped rabbit ventricular myocytes, Zygmunt and Gibbons showed the existence of a strongly outward rectifying $I_{Cl(Ca)}$ in the absence of $I_{NS(Ca)}$ at 22°C. They used step depolarizations and excitation-contraction coupling to evoke [Ca$^{2+}$]i, transients. Because of the highly synchronized local SR Ca$^{2+}$ release, this would be especially effective in activating Ca$^{2+}$-dependent currents. Laflamme and Becker confirmed a strongly outward rectifying $I_{Cl(Ca)}$ even during spontaneous SR Ca$^{2+}$ release in rabbit ventricular myocytes with no evidence of $I_{NS(Ca)}$ (again with $I_{Na/Ca}$ blocked and at 22°C). A marked increase of $I_{Cl(Ca)}$ can be seen at 35°C versus 25°C. Characteristics of $I_{Cl(Ca)}$ do support its apparent contribution to $I_m$ in APs (Figure 4A), despite its minor role (<10%) in the generation of DADs in our experiments (Figure 4B). These characteristics of $I_{Cl(Ca)}$ do support its apparent contribution to $I_m$. In contrast, Wu and Anderson observed a residual oscillatory current after blockade of both $I_{Cl(Ca)}$ and $I_{NS(Ca)}$, which was sensitive to the removal of extracellular cations. Although $I_{Cl(Ca)}$ may exist in rabbit myocytes, we find no evidence for its participation in DADs. Our findings are in contrast to a study by Szigeti et al, who inferred that $I_{Cl(Ca)}$ was the dominant Ca$^{2+}$-dependent inward current in rabbit ventricular, atrial, and Purkinje cells. In dog ventricular myocytes, $I_m$ appears to be carried almost equally by $I_{Na/Ca}$ ($\approx 60\%$) and $I_{Cl(Ca)}$ ($\approx 40\%$). This could be a species difference or due to their rapid SR Ca$^{2+}$ release. The highly synchronized SR Ca$^{2+}$ release would produce higher local [Ca$^{2+}$], which could better activate $I_{Cl(Ca)}$ (apparent $K_E = 150$ μmol/L [Ca$^{2+}$]). On the basis of hysteresis loops of [Ca$^{2+}$]i, versus $I_{Na/Ca}$ or $I_{Cr(Ca)}$, Trafford et al inferred a closer physical location of $I_{Cl(Ca)}$ to the ryanodine receptor than for $I_{Na/Ca}$ in ferret myocytes.

**Discussion**

In the present study, we characterized quantitatively for the first time the relationship between [Ca$^{2+}$]i, transients and membrane depolarization in a setting that mimics DADs, an important precursor of triggered arrhythmias. We show that DADs are almost entirely due to $I_{Na/Ca}$, whereas $I_{Cl(Ca)}$ and $I_{NS(Ca)}$ play little or no role in the generation of Ca$^{2+}$-induced $D_{E_m}$ at resting $E_m$. $D_{E_m}$-induced Ca$^{2+}$ release would produce higher local [Ca$^{2+}$], which could better activate $I_{Cl(Ca)}$ (apparent $K_E = 150$ μmol/L [Ca$^{2+}$]). On the basis of hysteresis loops of [Ca$^{2+}$]i, versus $I_{Na/Ca}$ or $I_{Cr(Ca)}$, Trafford et al inferred a closer physical location of $I_{Cl(Ca)}$ to the ryanodine receptor than for $I_{Na/Ca}$ in ferret myocytes.

**Relationship of [Ca$^{2+}$]i, Transient and DAD**

DADs are triggered depolarizations, seen in SR Ca$^{2+}$ overload, with spontaneous SR Ca$^{2+}$ release being the underlying event. In the present study, we used more controlled caffeine-induced SR Ca$^{2+}$ release (cDADs) and sacrificed the normal spontaneous nature of DADs. However, this control allowed systematic quantitative analysis of the Ca$^{2+}$ dependence of depolarization over a broad [Ca$^{2+}$]i range, which cannot be achieved by spontaneous SR Ca$^{2+}$ release. This also provides SR Ca$^{2+}$ load data that would not be available during propagating Ca$^{2+}$ waves associated with the spontaneous Ca$^{2+}$
release of Ca\(^{2+}\) overload. The trade-off we make for these big advantages is that we must separately consider the impact on E\(_m\), of spreading out the Ca\(^{2+}\)-induced current in time (as in slower Ca\(^{2+}\) waves). The current injection experiments in Figure 6 address this. That is, we cannot spread the Ca\(^{2+}\) transient in time (in a controlled and measured way), but we can do this with the resulting current (as pseudo I\(_S\)) to simulate a Ca\(^{2+}\) wave–induced DADs.

I\(_{Na,Ca}\) is approximately linear as a function of [Ca\(^{2+}\)],\(^3,4,36\) but the amount of depolarization produced is nonlinear because of interactions with other currents (eg, I\(_K1\), I\(_S\)) and membrane properties. However, we focus on the integrated E\(_m\) response because depolarization is the immediate cause of triggered APs due to SR Ca\(^{2+}\) release. An exponential equation describes well this Ca\(^{2+}\) dependence of \(\Delta E_m\) (with a doubling of DAD for every 88 nmol/L rise in [Ca\(^{2+}\)].)

The threshold of SR Ca\(^{2+}\) release that raises [Ca\(^{2+}\)], by 424 nmol/L, equivalent to an integrated I\(_{Na,Ca}\) of 0.89 C/F, is sufficient to trigger an AP with a threshold E\(_m\) of \(-65\pm1\) mV. This \(\Delta[Ca^{2+}]\), requires a total SR Ca\(^{2+}\) release of \(\approx50\) to 60 \(\mu\)mol/L cytosol, \(\approx50\%\) to 70% of the SR Ca\(^{2+}\) load. It is likely that at least this amount of Ca\(^{2+}\) is released during a spontaneous Ca\(^{2+}\) release under Ca\(^{2+}\)-overload conditions.\(^1,12\) However, several factors may limit this from triggering an AP in a normal cell. First, spontaneous SR Ca\(^{2+}\) release normally occurs as a wave,\(^5,13,17\) with [Ca\(^{2+}\)], not rising synchronously and consequently leading to a slower rise in Ca\(^{2+}\)-activated currents. Taking this into account \(\approx3\) times more \(\Delta[Ca^{2+}]\), may be required to trigger an AP under these conditions (on the basis of Figure 6). Second, even if all the SR Ca\(^{2+}\) is released during a wave, only 15% to 20% of the SR Ca\(^{2+}\) load is extruded from the cell via I\(_{Na,Ca}\)\(^1,12,38\). It is possible that at least some of this Ca\(^{2+}\) release is reaccumulated after the release channels close (unlike in our cDADs, where caffeine is present). This may limit the integrated I\(_{Na,Ca}\), more than peak I\(_{Na,Ca}\), but may nonetheless reduce the efficacy of a DAD leading to a triggered AP. Thus, the normal ventricular myocyte may have a reasonable safety margin of 3- to 4-fold against any SR Ca\(^{2+}\) release being able to trigger an AP.

An additional safety factor in the whole heart is that neighboring cells will act as current sinks, blunting the \(\Delta E_m\) effect for a given local I\(_{Na,Ca}\). However, cellular changes that cause either more SR Ca\(^{2+}\) release or greater local depolarization for a given \(\Delta[Ca^{2+}]\), increase propensity for triggered arrhythmias. That is, there would be greater chance for a cell cluster that is local enough, synchronous enough, and large enough to overcome the 3-dimensional current sink problem and trigger a propagating arrhythmia.

**Possible Arrhythmogenic Role in HF**

In HF, Na-Ca\(^{2+}\) exchange protein and I\(_{Na,Ca}\) can be doubled and I\(_{K1}\) is reduced by up to \(\approx50\%\),\(^2,18,19,39,41\) and may overcome the safety factor above. That is, doubling I\(_{Na,Ca}\) will double I\(_{Na}\) amplitude for any given SR Ca\(^{2+}\) release and reduction of I\(_{K1}\) by 50% will allow a given I\(_{Na}\) to be more effective in depolarizing the cell toward threshold for a triggered AP. Although SR Ca\(^{2+}\) load may be low in HF, adrenergic activity may increase Ca\(^{2+}\) load, allowing spontaneous Ca\(^{2+}\) release. Consequently, HF may greatly increase the propensity for DADs to trigger APs, with 4-fold less \(\Delta[Ca^{2+}]\), being sufficient to trigger an AP (as seen in computer models).\(^4\) Because triggered arrhythmias cause the majority of sudden cardiac death in nonischemic HF,\(^1\) it would be of vital interest to measure the \(\Delta[Ca^{2+}]\), dependence of \(\Delta E_m\) and the threshold in HF to test the above-mentioned working hypothesis.

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