Nonequilibrium Reactivation of Na⁺ Current Drives Early Afterdepolarizations in Mouse Ventricle

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Background—Early afterdepolarizations (EADs) are triggers of cardiac arrhythmia driven by L-type Ca²⁺ current (I_{CaL}) reactivation or sarcoplasmic reticulum Ca²⁺ release and Na⁺/Ca²⁺ exchange in large mammals. In contrast, EADs in the murine ventricle are driven by nonequilibrium reactivation of Na⁺ current (I_{Na}).

Methods and Results—In Tg myocytes, β-adrenergic challenge slowed late repolarization, potentiated sarcoplasmic reticulum Ca²⁺ release and initiated EADs below the I_{CaL} activation range (~47±0.7 mV). These EADs were abolished by caffeine and tetrodotoxin (but not ranolazine), suggesting that sarcoplasmic reticulum Ca²⁺ release and Na⁺/Ca²⁺ exchange shape late action potential repolarization to favor nonequilibrium reactivation of I_{Na}, and thereby drive the EAD upstroke. Action potential clamp experiments suggest that lidocaine eliminates virtually all inward current elicited by EADs, and that this effect occurs at concentrations (40–60 μmol/L) for which lidocaine remains specific for inactivated Na⁺ channels. This strongly suggests that previously inactive channels are recruited during the EAD upstroke, and that nonequilibrium I_{Na} dynamics underlie murine EADs.

Conclusions—Nonequilibrium reactivation of I_{Na} drives murine EADs. (Circ Arrhythm Electrophysiol. 2014;7:1205-1213.)

Key Words: arrhythmias, cardiac ■ calcium/calmodulin-dependent protein kinase type 2 ■ electrophysiology ■ sodium-calcium exchanger 1

It is generally accepted that lethal ventricular arrhythmias occur when a triggering event is able to propagate into electrophysiologically susceptible tissue.1 Premature ventricular contractions are prototypical triggers in the myocardium and are thought to emerge from arrhythmogenic cellular events known as early and delayed afterdepolarizations (EADs or DADs).2 DADs occur in the diastolic interval and result from interaction between I_{CaL} and the repolarizing K⁺ currents. However, EADs are also frequent in the rapidly repolarizing mouse action potential, which should not readily permit I_{CaL} reactivation. This suggests that murine EADs exhibit unique dynamics, which are key for interpreting arrhythmia mechanisms in this ubiquitous model organism. We investigated these dynamics in myocytes from arrhythmia-susceptible calcium calmodulin-dependent protein kinase II delta C (CaMKIIδC)-overexpressing mice (Tg), and via computational simulations.

Methods and Results—In Tg myocytes, β-adrenergic challenge slowed late repolarization, potentiated sarcoplasmic reticulum Ca²⁺ release and initiated EADs below the I_{CaL} activation range (~47±0.7 mV). These EADs were abolished by caffeine and tetrodotoxin (but not ranolazine), suggesting that sarcoplasmic reticulum Ca²⁺ release and Na⁺/Ca²⁺ exchange shape late action potential repolarization to favor nonequilibrium reactivation of I_{Na}, and thereby drive the EAD upstroke. Action potential clamp experiments suggest that lidocaine eliminates virtually all inward current elicited by EADs, and that this effect occurs at concentrations (40–60 μmol/L) for which lidocaine remains specific for inactivated Na⁺ channels. This strongly suggests that previously inactive channels are recruited during the EAD upstroke, and that nonequilibrium I_{Na} dynamics underlie murine EADs.

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Clinical Perspective on p 1213

In contrast, the murine ventricular AP repolarizes rapidly (tens of ms) and exhibits a brief plateau at negative

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1205
EADs ($-50$ mV). These characteristics sharply limit the potential for $I_{\text{CaL}}$ reactivation, yet numerous studies have observed EADs in the mouse,\textsuperscript{3–13} and most have implicated $I_{\text{CaL}}$.\textsuperscript{11–14} This presents a paradox on the electrophysiological conditions required to elicit EADs in the mouse ventricle, which has important implications for interpreting arrhythmia mechanisms in a large number of disease models. To clarify the dynamics capable of driving murine EADs, we have combined experimental and computational approaches to assess EAD mechanisms in myocytes from arrhythmia-susceptible calcium calmodulin-dependent protein kinase II delta C (CaMKIIδC)-overexpressing mice (Tg).\textsuperscript{15}

EADs appearing during $\beta$-adrenergic stimulation were initiated by nonequilibrium reactivation of $I_{\text{Na}}$ secondary to exaggerated SCR and $I_{\text{Na}^*}$. In some instances this evoked subsequent reactivation of $I_{\text{CaL}}$ and dynamical behavior similar to that described for larger species. However, the majority of events remained reliant on nonequilibrium $I_{\text{Na}^*}$ dynamics. Thus, we suggest that the mouse AP imposes unique constraints on EAD dynamics that rely on an $I_{\text{Na}}$-dependent mechanism of initiation.

Methods

A detailed description of all methods and analyses are provided in the Data Supplement.

CaMKIIδC Transgenic Mice

Male and female cardiac-specific CaMKIIδC transgenic mice (n=135) and WT littermates (n=130) were studied at 60±6.0 days of age (both groups). These mice have been described previously in detail\textsuperscript{13,16}; however, we note that they now exhibit a slower time course of heart failure development than originally published (Figure IV in the Data Supplement). The overall sample sizes permitted well-powered analyses for all designs. The number of animals was $>15$ to 25 per group for patch-clamp experiments where experimental success rates were relatively low. For field-pacing experiments, where cell yields per animal are much higher (typically 5–10), the number of animals for all groups is ≥25. For all designs we have attempted to account for the effect of interanimal variability (as distinct from intercell variability), by including individual animals as a random factor in our statistical model (see Statistics section of this article). All procedures received prior approval from the University of California San Diego Animal Subjects Committee.

Myocyte Isolation, Electrophysiology, and Ca$^{2+}$ Imaging

CaMKIIδC myocytes were isolated via enzymatic dissociation and stored for ≥22 hours at 37°C before undergoing 1 of 2 protocols: (1) current clamp AP recordings in whole-cell patch configuration or (2) pause-induced spontaneous Ca$^{2+}$ release under field-pacing (SCR). For patch-clamp experiments, simultaneous Ca$^{2+}$ imaging was performed via Fura-2 with internal solution containing (mM/μL) 120 K-aspartate, 10 KCl, 10 NaCl, 5 Mg-ATP, 1 MgCl$_2$, 0.3 Li-GTP, and 125 μmol/L Fura-2 potassium salt (pH 7.2, KOH). The external solution for both patch-clamp and field-pacing experiments (normal Tyrode’s (NT)) contained (mmol/L) 140 NaCl, 4 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 glucose, and 10 HEPES (pH 7.4, NaOH). Isoproterenol (Iso, 100 nmol/L) was added from frozen stock (1 mmol/L in 0.01N HCl), and caffeine (10 mmol/L) was prepared fresh from powder. For both experiments cells were preloaded with Ca$^{2+}$-sensitive dyes in 37°C media: Fura-2 μmol/L for patch-clamp (15 minutes, 5 μmol/L/L. Life Technologies: F1225) and Fluor-3 AM for SCR (40 minutes, 5 μmol/L/L. Life Technologies: F1241). Cells were then twice rinsed and stored in NT for 20 minutes to allow de-esterification. During current clamp experiments, cells were paced for 50 cycles (1 Hz) in NT before Iso was applied focally by microbore tubing. Another 50 cycles were acquired ≥1 minute after beginning Iso superfusion, as such all patch-clamp recordings are paired analyses. In SCR experiments, cells were paced for 30 seconds (again 1 Hz), and followed by a 30-second pause for SCR before rapid caffeine contracture (10 mmol/L in 0 Na+/0 Ca$^{2+}$ Tyrode’s). For SCR, Iso containing NT was applied to a separate group of cells from the same isolation. The bathing solution was maintained at 37°C for all experiments, and the experimenter was blinded to the genotype of the cells.

Computational Models

Our mouse ventricular cell model was a modification of the Bondarenko myocyte.\textsuperscript{17} Alterations were made primarily to permit implementation of acute and chronic effects of CaMKII. These included phosphoregulation of the ryanodine receptor, $I_{\text{Na}Ca}$, and late $I_{\text{CaL}}$, as well as transcriptional regulation of the transient outward current ($I_{\text{to}}$), inward rectifier current ($I_{\text{K1}}$), $I_{\text{Na}K}$, and sarco-endoplasmic reticulum Ca$^{2+}$-ATPase.\textsuperscript{16,20,21} $\beta$-adrenergic stimulation was simulated via established effects at $I_{\text{Na}}$, the ultrarapid delayed rectifier current ($I_{\text{Kur}}$), and on function of the Na$^+$/K$^+$-ATPase and SR Ca$^{2+}$-ATPase. We did not implement explicit protein kinase A (PKA)-dependent effects on $I_{\text{Kur}}$ gating, in part, because the effects are complex and overlap with those of CaMKII (which are included). PKA has been reported to alter voltage dependence of activation (to a variable extent)\textsuperscript{22,23} and inactivation (which may be mediated by CaMKII)\textsuperscript{23} and increase conductance (which may be because of translocation of channels to the membrane, requiring >5 minutes).\textsuperscript{22,24} A trafficking effect is unlikely to occur in our 2-3 minute time course, inactivation effects are included (as part of the CaMKII module), and any negative shifts in $I_{\text{Kur}}$ activation would tend to enhance the EADs observed in our simulations.

Statistics

Fisher exact test was used for EAD susceptibility in terms of proportion of cells. The Kruskal–Wallis test was used for percent of cycles with EADs or DADs. This test was applied across all 4 groups (WT, Tg, WT+Iso, and Tg+Iso), and followed by either Mann–Whitney (unpaired data) or Wilcoxon signed-rank (paired data) post hoc tests, with Bonferroni correction. Because of larger sample sizes for SCR experiments, statistical significance for incidence of SCR was estimated by $\chi^2$ tests, and mean event frequencies (events/cell) were assessed by the Mann–Whitney test. For the SCR experiments in particular, the number of cells contributed per animal was relatively high (typically 5–10), and it is possible that interanimal variation contributed to the effects we have otherwise attributed to genotype in the above statistical procedures. As such, we also performed a series of mixed models (for both EAD and SCR datasets) in which the variability introduced by individual animals was included as a random factor in addition to the fixed factors of genotype (WT, Tg), drug (NT, Iso), and the genotypexdrug interaction. These analyses yielded remarkably similar results to the more common approaches described above, and only 1 slight discrepancy was observed for SCR incidence (see Results section of this article). Repeated measures ANOVA was used for AP and Ca$^{2+}$-handling comparisons after separating the transgenic cells into EAD+ and EAD– subsamples (see Results section of this article). Student’s $t$ tests were applied with Bonferroni correction for all post hoc comparisons. All analyses were performed using the R software package (version 3.1.1).

Results

Cellular Arrhythmogenesis in CaMKIIδC Transgenic Myocytes

The cardiac-specific CaMKIIδC transgenic mouse develops heart failure, is arrhythmia-susceptible, and presents increased cellular susceptibility to both EADs and DADs.\textsuperscript{10,16,20,21} (Figure IV in the Data Supplement). Here, we elicited EADs through...
β-adrenergic challenge by Iso (100 nmol/L; Figure 1A and 1B), and while DADs and SCR were almost completely absent in patch-clamp experiments (19 events in 1881 cycles, Tg versus WT: P=0.34), Tg myocytes were more susceptible to paused-induced SCR during Iso treatment. The number of pause-induced events per cell was higher for Tg cells, particularly during Iso challenge (P<0.05; Figure 1C), and SCR incidence also tended to be higher. This effect was significant when assessed by χ² analyses (P=0.041), but not with logistic regression via the mixed model described above (P=0.097; Figure 1C). Importantly, the ability of Iso to induce both arrhythmogenic behaviors was associated with normalization of a baseline deficit in SR Ca²⁺ content in Tg cells (Figure 1C, bottom right). This suggests that an Iso-induced increase in SR Ca²⁺ load may be central to both forms of electrophysiological instability.

Enhanced Ca²⁺ Handling and Prolonged Repolarization Precede EAD Initiation

To describe the changes in steady-state Ca²⁺ handling and AP morphology associated with EADs, we separated the Tg cells into those that did exhibit EADs (EAD+, n=12) and did not exhibit EADs (EAD−, n=15) in ≥10% of cycles. These 2 Tg cell populations were compared with all WT cells (n=17). In EAD+ recordings, steady-state data were taken from the 5 to 10 cycles immediately before the first EAD, for all other cells we used the final 5 to 10 cycles of the recording. These analyses suggest that murine EAD susceptibility is associated with exaggerated Ca²⁺ cycling and slowed late repolarization immediately before EAD initiation (Figure 2). At baseline (NT), Ca²⁺ transients were smaller and slower to decay in EAD+ cells compared with WT. The baseline AP in EAD+ cells also exhibited slower early repolarization, although late repolarization was not different from WT (P=0.13). As expected, Iso increased Ca²⁺ transient amplitude and accelerated Ca²⁺ decay in WT cells. These effects were exaggerated in EAD+ cells such that, immediately before initiation of EADs, Iso had normalized the baseline deficits in both Ca²⁺ transient amplitude and decay rate. This resulted in an Iso-induced increase in transient amplitude that was more than doubled in EAD+ cells relative to WT (Figure 2A, bottom right, and Figure 2B, top right). AP prolongation accompanied these Iso-dependent Ca²⁺ handling changes in both WT and Tg cells, and again this effect was amplified in EAD+ cells, particularly for late repolarization where AP duration at 90% repolarization (APD₉₀) was 53% longer than in Iso-treated WT myocytes (P<0.05).

SCR Is Required for Murine EADs

These steady-state effects suggest that Iso elicits EADs in Tg cells by enhancing Ca²⁺ cycling in a manner that destabilizes repolarization. However, all observed Ca²⁺ handling and AP changes could be because of gain-of-function regulation at either I₅ᵥ or SCR, both of which are recognized consequences of CaMKII activation⁴⁻⁶,⁰⁻⁴ and are the primary drivers of EADs in large mammals.⁷⁻⁷,²⁷
The low late AP plateau in Iso (Figure 2B, top) is probably because of greater SCR and prolonged inward $I_{NaCa}$, as suggested by earlier investigations. To test whether this plays a role in driving the observed EADs, we rapidly applied caffeine to a cell exhibiting Iso-induced EADs. This maneuver simultaneously eliminates inward currents driven by SCR and potentiates $I_{CaL}$ by reducing Ca$^{2+}$-dependent inactivation, thus exaggerating any $I_{CaL}$-dependent EAD mechanism. Figure 3 shows that caffeine caused the expected large Ca$^{2+}$ release and thereby triggered an AP. The sustained elevation in inward $I_{NaCa}$, which suspends terminal repolarization. The changes in AP morphology resulting from caffeine also support the contention that SCR promotes EADs by altering the trajectory of late repolarization. Figure 3C shows that caffeine elicits inverse effects on early and late repolarization. APD$_{90}$ is dramatically increased, presumably because of less Ca$^{2+}$-dependent inactivation of $I_{CaL}$, whereas the $I_{NaCa}$-driven late plateau is removed (with repolarization parallel to the baseline AP trajectory). Thus, Iso causes larger SCR and inward $I_{NaCa}$, which suspends terminal repolarization.

**Unique Dynamics of Murine EADs**

The observed EADs could be discriminated by whether they exhibited sustained Ca$^{2+}$ transients, and we reasoned that this may indicate 2 populations of EADs caused by distinct mechanisms. Of the 198 EAD-containing cycles, 20.7% exhibited a sustained Ca$^{2+}$ transient type 1 EAD morphology (Figure 4A, left). The remaining EADs (79.3%) exhibited normal monotonic Ca$^{2+}$ transient decay, and shorter $E_{m}$ plateaus with fewer oscillations (Figure 4A, right), type 2 EADs. Importantly, the take-off potentials of the first oscillations in both types were below the $I_{CaL}$ activation range, at $-46.4$ and $-47.7$ mV for types 1 and 2, respectively (Figure 4B, right). This further indicates that $I_{CaL}$ reactivation is unlikely to contribute to EAD initiation in either type of event. For type 1 EADs, subsequent oscillations took off from more positive potentials, whereas they became progressively more negative in type 2 EADs. Together, these observations indicate 2 characteristics of murine EADs that distinguish them from EADs in large mammals: (1) the initiating event relies on dynamics that cannot be explained by $I_{CaL}$ reactivation and (2) although $I_{CaL}$ reactivation may contribute to EAD maintenance in cycles with sustained Ca$^{2+}$ transients (type 1), a separate dynamical mechanism can support multiple oscillations in type 2 EADs.

Temporal changes in oscillation amplitude and period provide further support for the existence of multiple dynamical mechanisms in the mouse. A signature of $I_{CaL}$-dependent EAD dynamics in large mammals is that, within a given AP, EAD oscillations increase in both amplitude and period before spontaneously terminating. These properties have been described in terms of nonlinear dynamics, which suggest that the $I_{CaL}$ and $K^+$ current interactions evolve through a destabilizing Hopf bifurcation, from which oscillation amplitude and period increase before terminating at a homoclinic bifurcation. Figure 4C (left) shows that, although both types of EAD exhibit similarly large initiating oscillations, only type 1 EADs also present the characteristic increase in amplitude from the midpoint oscillation to the final oscillation of the cycle. Oscillation period increases from the first oscillation to the last in both types of EAD, although the first oscillation is more rapid in the type 2 events compared with type 1 (Figure 4C, right). In sum, these analyses suggest that the 2 types of EAD are initiated by a common dynamical mechanism that does not require $I_{CaL}$, but that type 1 EADs then recruit $I_{CaL}$ and...
exhibit late dynamics that are similar to those observed in larger mammals.

**SCR Slows Late Repolarization and Recruits I \(_{Na}\) to Initiate EADs**

Because the mechanism of EAD initiation requires SCR and occurs at negative potentials that favor forward-mode Na+/Ca\(^{2+}\) exchange, I \(_{Na,\text{Ca}}\) is an obvious candidate for driving EAD initiation. However, it is unlikely that the conventionally small, slow, and monotonically decaying forward-mode I \(_{Na,\text{Ca}}\) could elicit the large and fast depolarizations observed here. Thus, it is more likely that Ca\(^{2+}\) release and I \(_{Na,\text{Ca}}\) contribute by conditioning late repolarization to permit reactivation of another major inward current. To interrogate these dynamics quantitatively, we developed a computational model of the mouse ventricular myocyte (see Methods section and Data Supplement for details of this implementation).

The EADs produced by this model recapitulated the more frequent type 2 EAD morphology. Figure 5A shows a sequence of simulated beats in ISO showing transition through action potential (AP) prolongation to EAD initiation as whole-cell Ca\(^{2+}\) load and sarcoplasmic reticulum (SR) Ca\(^{2+}\) release increases. B, Simulations of the first EAD+ beat in the model where specific components were deleted (only in this beat) to test their acute effects in EAD initiation. The deleted components were (4) SR Ca\(^{2+}\) release (G\(_{\text{Ca,SR}}\)=0); (5) I \(_{Na,\text{Ca}}\) (V\(_{\text{NCX,Ca}}\)=0); (6) calcium calmodulin-dependent protein kinase II (CaMKII) effects at I \(_{\text{Ca,L}}\) (G\(_{\text{CaL}}\)=0.3458, K\(_{\text{pc,max}}\)=0.1162). C, Four APs and corresponding inward currents, showing the role of I \(_{Na}\) in both AP prolongation and EAD initiation.

**Figure 5.** I \(_{Na}\) drives early afterdepolarization (EAD) initiation in murine ventricular myocytes. A, A sequence of simulated beats in ISO showing transition through action potential (AP) prolongation to EAD initiation as whole-cell Ca\(^{2+}\) load and sarcoplasmic reticulum (SR) Ca\(^{2+}\) release increases. B, Simulations of the first EAD+ beat in the model where specific components were deleted (only in this beat) to test their acute effects in EAD initiation. The deleted components were (4) SR Ca\(^{2+}\) release (G\(_{\text{Ca,SR}}\)=0); (5) I \(_{Na,\text{Ca}}\) (V\(_{\text{NCX,Ca}}\)=0); (6) calcium calmodulin-dependent protein kinase II (CaMKII) effects at I \(_{\text{Ca,L}}\) (G\(_{\text{CaL}}\)=0.3458, K\(_{\text{pc,max}}\)=0.1162). C, Four APs and corresponding inward currents, showing the role of I \(_{Na}\) in both AP prolongation and EAD initiation.

**Figure 6.** Nonequilibrium reactivation of I \(_{Na}\) carries the early afterdepolarization (EAD) upstroke. A, EADs were interrupted first by rapid application of 10 \(\mu\)mol/L ranolazine, after washout, tetrodotoxin (TTX) was progressively introduced to the bath and eliminated EADs at 1 \(\mu\)mol/L. B, (a) The states of the INa model: C1–C3 are closed states, LC1–LC3 are burst-mode closed states, IF is the fast inactivation state, I1 and I2 represent intermediate and deeply inactivated channels, and finally, O and LO are the normal and burst-mode open states, respectively. (b) EADs were eliminated by simulated 1 \(\mu\)mol/L TTX (50% reduction in G\(_{\text{Na}}\)). C, The time course of LO and O occupancies during a simulated EAD, where the canonical open state (O) repopulates before the late open state LO and supports a role for nonequilibrium reactivation of INa in EAD initiation. C, (a) lidocaine blockade during action potential (AP) clamp of a wild-type (WT) mouse myocyte indicates that a substantial lidocaine-sensitive current is recruited by the EAD upstroke. Top, The clamped AP waveform (collected in a Tg cell); middle, the corresponding raw current (I\(_{\text{out}}\)); and bottom, the lidocaine-sensitive difference current (I\(_{\text{lidocaine}}\)). Lidocaine concentrations are shown as inset. (b) The same experimental AP waveform is applied to the WT INa model and yields similar current reactivation.

either SCR (4 versus 3) or I \(_{Na,\text{Ca}}\) (5 versus 3) did eliminate EADs. Notably, I \(_{Na,\text{Ca}}\) elicits both AP prolongation and EAD initiation via recruitment of INa, which exhibits a progressive induction and then clear bifurcation (Figure 5C) as Ca\(^{2+}\) transient amplitude, SCR, and I \(_{Na,\text{Ca}}\) increase. Although I \(_{Na,\text{Ca}}\) is well known to mediate Ca\(^{2+}\)-dependent modulation of late AP duration in the mouse, this interaction between I \(_{Na,\text{Ca}}\)
and I_{Na} reactivation is not widely appreciated as contributor to AP prolongation in the mouse. However, these simulations suggest that, even prior to EAD initiation, inward current contributed by reactivating I_{Na} is responsible for much more of the observed AP prolongation than I_{NaCl} (Figure 5C, first and second columns). I_{CaL} was also reactivated to a small extent during EADs, but this is secondary to I_{Na}. We were unable to find an EAD regime that replicated the less frequent type 1 EAD morphology, and we suggest that this behavior may require a more complex representation of EC coupling than is present in this model, which does not permit permitted or reactivating SCR (see RyR section in the Data Supplement).

EAD Initiation Is Carried By Nonequilibrium Reactivation of I_{Na}

To experimentally test the model prediction that I_{Na} is responsible for driving EAD initiation, we attempted to interrupt Iso-induced EADs by applying I_{Na} inhibitors of differing selectivity for the late and fast components of I_{Na}. In the same cell we first rapidly applied 10 μmol/L ranolazine, and after brief washout, introduced tetrodotoxin at 100 nmol/L, 1 μmol/L, and 5 μmol/L (Figure 6). Ranolazine exhibits greater selectivity for I_{Na}, and at 1 Hz, 10 μmol/L ranolazine achieves ~70% attenuation of I_{Na}, but near negligible inhibition of peak I_{Na} (100 nmol/L). Figure 6A shows that, although this treatment clearly inhibited prolonged EADs, it did not prevent EAD initiation (trace 2 versus 1). Similarly, tetrodotoxin at a dose capable of inhibiting neuronal I_{Na} (100 nmol/L) did not prevent the EAD upstroke (Figure 6A, trace 3). However, tetrodotoxin eliminated EADs at a dose (1 μmol/L) approximately sufficient for half inhibition of peak myocardial I_{Na} and 30% inhibition of I_{Na} (Figure 6A, traces 4 and 5; full recordings are provided in Figure V in the Data Supplement). The ability of tetrodotoxin to eliminate EADs was reproducible; 3 of 3 cells were returned to normal AP morphology on rapid application of tetrodotoxin (Figure VI in the Data Supplement). Thus, although I_{Na} may contribute to the dynamics of plateau EADs by contributing inward current late in the AP, this component of the Na+ current is not responsible for initiating murine EADs. Instead it seems that EAD initiation is carried by reactivation of canonical fast I_{Na}.

Although these reactivations initiate above the E_{m} range that typically permits steady-state I_{Na} availability, nonequilibrium reactivation of myocardial I_{Na} is known to occur in both normal and pathologically mutated NaV1.5 channels. Returning to our model, we confirmed that an acute 50% reduction in I_{Na} conductance eliminated EADs as in the experiments (Figure 6B (b)), and observed that the state occupancies of the I_{Na} model support the contention that nonequilibrium I_{Na} reactivation is responsible for EAD initiation. Figure 6B (c) shows that the fast open state (O) repopulates before the burst-mode open state (LO) and achieves ~5× greater peak state occupancy during the EAD upstroke. These channel reopenings are fueled by slight recovery through the canonical closed states (C1–C3, Figure VII in the Data Supplement).

To test this model result experimentally we performed AP clamp experiments with one of the recorded EAD waveforms in the presence of various concentrations of lidocaine. This I_{Na} antagonist is largely selective for inactivated channels, and as shown in Figure 6C (a) it is clear that the EAD upstroke in these AP clamps induces a significant lidocaine-sensitive inward current, even below the concentration range that inhibits noninactivated channels. Furthermore, applying the same experimental AP waveform to clamp the I_{Na} model achieves similar reactivation characteristics (Figure 6C (b)). Importantly, these reactivation dynamics were not attributable to the modeled effects of CaMKII at the Na' channel. Rather the established leftward-shift in steady-state inactivation, and slower recovery from inactivation, both of which are incorporated into the Tg I_{Na} model, serve to limit nonequilibrium reactivation. Figure 7A shows that reactivation is exaggerated in the isolated WT I_{Na} model compared with the Tg model during AP clamp of the first Tg EAD waveform. As described previously, the propensity for this reactivation is highly dependent on the trajectory of repolarization.

To investigate whether differences between the mouse and large mammal APs should be expected to alter the impact of CaMKII on these reactivation dynamics, we applied repolarizing voltage ramps of differing trajectories to the I_{Na} models. As shown in Figure 7B, a slowly repolarizing ramp (~1 V/s) after prolonged depolarization (Figure 7B (a)) elicited much less nonequilibrium reactivation than more rapid...
The role of SCR in EAD dynamics has been appreciated for >20 years. In large mammals, triggered Ca²⁺ release is thought to promote EADs in 1 of 2 ways: (1) potentiated, but still synchronous, Ca²⁺ release and inward I_{NaCa} is sufficient to disrupt the balance of currents during the AP plateau, and thereby condition repolarization to enter the low plateau, and thereby condition repolarization to enter the slow ramp, but these events still require initiation by the Ca²⁺ release triggered by excitation. It is possible that spontaneous Ca²⁺ waves exhibit slower kinetics, whereas spontaneous Ca²⁺ waves exhibit slower kinetics, which again drives I_{NaCa} and may recruit accessory inward currents. Although we have not assessed the potential for Ca²⁺ waves to contribute to the EADs observed here, the time-scale on which these events develop suggest that they are unlikely to have contributed to EAD initiation. The first EAD oscillation typically occurred between 30 and 50 ms after the triggering current pulse in our experiments, whereas spontaneous Ca²⁺ waves exhibit slower kinetics, in which peak [Ca²⁺] develops over hundreds of milliseconds. Thus, any spontaneous event not detected before the AP was stimulated would quickly be overwhelmed by the Ca²⁺ release triggered by excitation. It is possible that wave-like dynamics contribute to plateau oscillations in type 1 EADs, but these events still require initiation by the faster dynamical mechanism. Thus, we contend that the suspended repolarization because of sustained I_{NaCa} is more likely to describe how SCR contributes to EAD dynamics in the mouse. This mechanism also explains how gain-of-function changes to I_{Ca,L} gating and expression can promote murine EADs without eliciting I_{Ca,L} reactivation, that is, by potentiating SCR through increased SR Ca²⁺ load and fractional release. Because Ca²⁺ channel blockers would indirectly prevent the I_{NaCa}-dependent low plateau, and may have off-target effects, we avoided such experiments. Thus, we also strongly recommend against interpreting the ability of I_{Ca,L} inhibitors to reduce EAD incidence in the mouse as inferring a role for I_{Ca,L} reactivation in driving murine EADs.

Although cardiac EADs have most commonly been associated with the late component of I_{Na}, previous studies have also recognized that reactivation of fast I_{Na} may be involved in certain contexts. Most clearly, the LQT3 mutation I1768V exhibits exaggerated nonequilibrium reactivation during slow ramp repolarization, but seems innocuous by standard square-pulse characterization. Simulation of these properties within the Luo-Rudy guinea pig ionic model recapitulated AP prolongation at normal sinus rates and yielded EADs in simulated bradycardia. More generally, early studies suggested that reactivation of I_{Na} may drive EADs initiating at more negative potentials in large mammals. However, these mechanisms were quickly thought to be less physiologically important because they required more severe conditions to be induced, as might be expected of any mechanism that depends on I_{Na} recovery after the prolonged AP plateau. Together, these prior studies suggest that, in large mammals, the conditions required to achieve nonequilibrium reactivation of I_{Na} are probably supraphysiological in all but a few disease contexts. In contrast, the triangular mouse AP both accelerates recovery of I_{Na} and reduces the potential for I_{Ca,L} reactivation, hence shifting EAD dynamics to favor reactivation of I_{Na}. As a result, we conclude that, for any conditioning stimulus (exaggerated SCR and I_{NaCa} in the CaMKIIδC transgenic model used here), nonequilibrium I_{Na} dynamics are the most likely basis for EAD initiation in the mouse. Furthermore, our simulations suggest that these I_{Na} dynamics are responsible for the majority of late AP prolongation in the mouse under conditions of exaggerated Ca²⁺ cycling, particularly β-adrenergic challenge. This finding may be important for many murine simulation studies, where I_{Na} models that are not capable of representing nonequilibrium reactivation are unlikely to capture Ca²⁺-induced AP prolongation within reasonable constraint of I_{NaCa} and other currents that are active late in the AP.

A key question for the general importance of these findings is whether nonequilibrium reactivation is facilitated by CaMKII, and thus, whether this behavior may be specific to this Tg mouse model. To date, no data are available to describe a role for CaMKII in nonequilibrium reactivation, and we have not assessed it experimentally in this study. However, the loss-of-function effects that CaMKII exerts on steady-state I_{Na} would be expected to impair rather than facilitate nonequilibrium reactivation, and our simulations in the WT and Tg I_{Na} models support this assertion (Figure 7). Thus, although investigation of the potential for CaMKII to alter nonequilibrium gating of I_{Na} is warranted, there is currently little reason to expect such an outcome. Rather we think that these I_{Na} dynamics are a general property of murine electrophysiology, which depends on the trajectory of AP repolarization rather than CaMKII-dependent alteration of I_{Na} gating properties.

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References

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**CLINICAL PERSPECTIVE**

Early afterdepolarizations are 1 of 3 forms of arrhythmogenic cellular event thought to underlie triggered arrhythmia in both atrial and ventricular myocardium. These events occur during repolarization of the action potential (AP), and in the human ventricle they are generally carried by reactivation of the L-type calcium current. However, under certain conditions or in cell types in which the AP is markedly shortened, there is relatively little opportunity for L-type channels to reactivate because membrane potential quickly descends through the voltage range in which they are active. In this study, we have used mouse ventricular cardiomyocytes (which have brief AP duration) and discovered that reactivation of sodium current is the dominant mechanism of early afterdepolarization initiation. This mechanism generally occurs in response to enhanced calcium cycling, such as that accompanying adrenergic challenge, and has 2 important implications. First, it imposes a constraint for interpreting early afterdepolarization mechanisms in cardiac myocytes which exhibit short APs with negative plateaus (eg, rat and mouse cardiac myocytes and human atrial myocytes). Second, it suggests a novel early afterdepolarization mechanism, and possibly a novel mechanism of triggered arrhythmia, in these tissues with rapidly repolarizing APs.
Nonequilibrium Reactivation of Na\(^{+}\) Current Drives Early Afterdepolarizations in Mouse Ventricles
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1 Experimental Methods

1.1 Myocyte isolation:

Myocytes were isolated from the LV and septum essentially as previously\(^\text{17}\). Collagenase concentration was 2 mg/mL (type 2, Worthington), and BDM was excluded throughout. Briefly, animals were heparinized (10 IU ip) 10-15 minutes before the heart was excised under isoflurane anaesthesia (3%). The heart was retrograde perfused under constant flow (\(~3\) mL/min), with nominally Ca\(^{2+}\) free solution (mmol/L: 113 NaCl, 4.7 KCl, 0.6 KH\(_2\)PO\(_4\), 0.6 Na\(_2\)HPO\(_4\), 1.2 MgSO\(_4\), 12 NaHCO\(_3\), 10 KHCO\(_3\), 10 hepes, 5.5 D-glucose, and Taurine 30). After 5 minutes, the perfusate was switched to enzyme and Ca\(^{2+}\) (12.5 \(\mu\)mol/L) containing solution for a further 15 minutes. The heart was then cut down, teased apart, and triturated in collagenase-free solution supplemented with calf serum (FCS, 5%). Cells were plated in minimum essential media (0.5% FCS) on laminin coated (1 \(\mu\)g/cm\(^2\) ) glass coverslips. Once seeded, cells were stored at 37\(^\circ\) C in serum-free media (0.5 mM Ca\(^{2+}\), pH 7.4) for use within the following 2 - 8 hours.

1.2 Experimental solutions:

As described in the Methods, the external solution (Normal Tyrode’s, NT) for all myocyte experiments contained (mmol/L) 140 NaCl, 4 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 D-glucose, and 10 Hepes (pH: 7.4, NaOH). Isoproterenol (100 nmol/L) was added from frozen stock (1 mmol/L in 0.01N HCl), and caffeine (10 mmol/L) was prepared fresh from powder. The internal solution contained (mmol/L) 140 K-Aspartate, 10 KCl, 10 NaCl, 5 Mg-ATP, 1 MgCl\(_2\), 0.3 Li-GTP, and 125 \(\mu\)mol/L Fura-2 potassium salt (pH 7.2, KOH).

1.3 Patch-clamping:

Whole-cell current-clamping was performed on visually quiescent, rod-shaped myocytes, with clear striations. High-resistance patch microelectrodes (8-13 MOhm with this internal solution) were pulled from borosilicate glass and used in fast I-clamp mode of an Axopatch 200B amplifier, controlled by pClamp 6 software and a Digidata 1200A interface. Membrane potential (\(E_m\)) was sampled at 5 kHz and Bessel filtered at 1 kHz. Measured (\(~10\) mV) liquid junction potential was used for \textit{a priori} correction of \(E_m\).
Fast capacitance was corrected in the cell-attached configuration. After gaining access, series resistance \( R_s \), which averaged 18.9 MOhms and was not different between Tg (19.2 ± 1.0) and WT (18.2 ± 1.2), was corrected for pacing current injection (1-2 ms, 0.5-2 nA square-wave pulses). Cells were then paced for 50 cycles (1 Hz) in NT before Iso was applied focally by 250 micron diameter micro-bore tubing. Another 50 cycles were acquired at least 1 minute after beginning Iso superfusion. The bathing solution was maintained at 37°C throughout, and the experimenter was blinded to the genotype of the cells.

1.4 Ca\(^{2+}\) epifluorescence:

**Fura-2:** As noted above, the internal solution contained free Fura-2 to limit dye diffusion into the pipette. An Ion Optix fluorescence system equipped with optics appropriate for Fura-2 (340/380 nm excitation, 510 nm emission) was interfaced with the patch-clamp acquisition system to synchronize the \( E_m \) and fluorescence signals. Initial F340/F380 was calculated after background subtraction at each excitation wavelength, and then 1st order Savitzky-Golay filtered to give the final ratio time-series.

**Fluo-3:** Fluo-3 fluorescence was excited at 480 nm and 535 nm emission was collected at 1 kHz. All recordings were background subtracted and normalized to mean diastolic Ca\(^{2+}\) concentration \( (F_0) \) during the 1 Hz pacing period.

1.5 Echocardiography:

Short-axis M-mode echocardiograms were recorded with an Agilent Technologies Sonos 5050 system and S12 pediatric probe (12 MHz, Philips). Animals were isoflurane anesthetized (1 - 1.5% maintenance) and warmed by a circulated water pad. M-mode data were collected at a depth of 2 cm, a sweep speed of 150 mm/s, and averaged over at least 3 full cycles. All data collection and analyses were performed by the same trained technician.

1.6 ECG:

Mice were tribromoethanol (2.5%) anesthetized, warmed, and instrumented for 3-lead ECG (limb needle electrodes) and Isoproterenol (Iso) challenge by tail vein infusion. The pseudo-lead 1 signal was externally conditioned by a Humbug noise cancellation unit (Quest Scientific, Vancouver), and recorded at 10 kHz. After 2 minutes of baseline recording, Iso (25 µg/kg) was administered in warmed physiologic saline by low volume (~ 0.05 cc) bolus infusion over 20 seconds, and the recording was continued for a further 8 minutes. Steady state ECG parameters were measured from 10-second epochs occurring at the beginning of minutes 1, 3, 5, 7, and 9, by custom Matlab software. For each epoch, all cycles (\( n \sim 60 \) to 100) were temporally aligned by the peak of the R-wave and averaged to give a smooth mean ECG waveform. Features of interest were manually identified by the same blinded analyst. The rate-corrected QT interval was calculated as by Mitchell et al. 1998\(^{16}\): \( QT_c = Q-T_0/(R-R_0/100)^{1/2} \). PVCs and other arrhythmia were visually identified by
a blinded analyst over the entire time-course. Ventricular tachycardia (VT) was defined as > 4 PVCs in sequence, and separated into non-sustained (< 30 s), and sustained episodes (≥ 30 s). Because very few ectopic events were observed under Iso challenge alone, a separate cohort received intraperitoneal injection of both caffeine (120 mg/kg) and epinephrine (2 mg/kg).

1.7 Analyses:
Early (EADs) and delayed afterdepolarizations (DADs) were identified by the same blinded analyst. Recordings for which EADs occurred in more than 10% of cycles were defined as EAD+, no recording exhibited DADs in more than 5% of cycles. In addition to conventional EAD and DAD morphologies, we observed a number of sustained events (SEs) that lasted 3 or more pacing cycles - Figure S9. Like DADs, these were infrequent (24 events in total), but unlike DADs they were more frequent in Tg cells during Iso challenge. Roughly equal numbers of SEs initiated spontaneously (11/24) as were triggered via the patch electrode (13/24). Because triggered SEs were indistinguishable from conventional EADs other than that they failed to terminate within one full cycle, we considered them a special class of EAD for all analyses. For each cell, steady state action potential and Ca$^{2+}$ transient characteristics were averaged over the final 5-10 stable cycles for each of the Iso and baseline recordings. In EAD+ recordings this period directly preceded initiation of EADs. For pause-induced SCR experiments, SCR events were manually identified by the same blinded analyst. SCR events less than 10% of the pacing Ca$^{2+}$ transient amplitude were thresholded out of all further analyses.

2 Computational mouse model:
The objective of our mouse myocyte model was to identify the current carrier/s likely to be responsible for EAD initiation. The base electrophysiologic model was the Bondarenko mouse myocyte$^5$, including recent updates$^4$. We modified this model either to improve general functional characteristics, or permit more straightforward fitting to our data. First we replaced several key Ca$^{2+}$ handling mechanisms and reparameterized a number of other sarcolemmal current carriers to better reflect established characteristics of murine Ca$^{2+}$ handling, improve model stability, or emulate our experimental conditions (e.g. physiologic temperature). Second, we parameterized this new Baseline model to yield a transgenic (Tg) model fit to data presented here or published previously. Lastly, we imposed a further parameter set to represent established effects of acute β-adrenergic challenge. The final parameter changes applied to each of these three stages of development are provided in Table 1.
2.1 Baseline modifications

The Bondarenko model provides a detailed foundation for this study, and was recently extended by Li et al.\textsuperscript{11}. We adopt several of their alterations, but have also diverged in a number of places to better fit data specifically collected at 1 Hz in CaMKII\delta\textsuperscript{C}-transgenic cells.

Our major changes were made to correct Ca\textsuperscript{2+}-handling mechanisms and achieve appropriate Ca\textsuperscript{2+} flux balance. We have replaced the original formulations of $I_{\text{NaCa}}$, SERCA, RyR, and $I_{\text{Na}}$. Our $I_{\text{NaCa}}$ implementation is a modified version of the Weber et al. model (W-B)\textsuperscript{21}. The models of SR Ca\textsuperscript{2+} release and reuptake are slight deviations from those of the Shannon-Bers myocyte (S-B)\textsuperscript{19}, and the $I_{\text{Na}}$ model is identical to that developed by Grandi et al.\textsuperscript{7} (G-B), and was chosen to incorporate CaMKII-specific regulatory effects at this current.

As in the L-S model, we also reparameterized the plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA) and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, to reduce overall Ca\textsuperscript{2+} extrusion to between 0.5 and 1\%, and prevent progressive Na\textsuperscript{+} overload, respectively. These and other simple parameter changes are presented in supplemental table 1.

2.1.1 Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange

Our goal in reimplementing $I_{\text{NaCa}}$ was to simultaneously match our experimental $[\text{Ca}\textsuperscript{2+}]_i$ decay kinetics, maintain realistic murine flux distribution between NCX and SERCA, and permit $[\text{Ca}\textsuperscript{2+}]_i$-dependent regulation of APD\textsubscript{90}. We have taken an approach similar to Livshitz et al.\textsuperscript{12}, who distributed a fraction ($\beta$) of the recruitable $I_{\text{NaCa}}$ to the same functional compartment as $I_{\text{CaL}}$ and SR Ca\textsuperscript{2+} release. Those authors used this definition to account for the well-known effects of intracellular Ca\textsuperscript{2+} gradients upon $I_{\text{NaCa}}$ and $I_{\text{CaL}}$ inactivation kinetics. We chose it both to provide some functionalistic account for these gradients during the AP, and to retain simple parameterization of $I_{\text{NaCa}}$ (2 free parameters). Finally, as in Li et al.,\textsuperscript{11} and because $[\text{Ca}\textsuperscript{2+}]_i$-dependent regulation was not originally observed in intact mouse myocytes, we did not include the allosteric term of the original W-B model\textsuperscript{21}. We note that this form of regulation has been observed in murine excised patches\textsuperscript{15}, and that this discrepancy remains unresolved, but may be explained by a higher affinity of the cytosolic regulatory site in the mouse, which would leave allosteric regulation near-saturated at diastolic $[\text{Ca}\textsuperscript{2+}]_i$.\textsuperscript{21} Our final formulation was:

$$I_{\text{NaCa}} = V_{\text{NCX}} \left( \beta \Delta E_{ss} + (1 - \beta) \Delta E_i \right)$$  \hspace{1cm} (1)

where $\beta$ is the fraction of NCX sensing dyadic subspace $[\text{Ca}\textsuperscript{2+}]_i[\text{Ca}\textsuperscript{2+}]_{ss}$, and both $\Delta E_{ss}$ and $\Delta E_i$ represent the electrochemical contributions to $I_{\text{NaCa}}$ resulting from the subspace and bulk myoplasmic...
concentrations, respectively. These are calculated as by Weber et al.\textsuperscript{21}, where $x$ refers to either $ss$ or $i$:

$$
\Delta E_x = \frac{([Na]^3_i[Ca]_o^{e^{\sigma VF}} - [Na]^3_i[Ca]_s^{e^{\sigma VF}})}{(K_{mCa}^3[Na]^3_i + K_3^{mNa} [Ca]_s + K_3^{mNa} [Ca]_o (1 + \frac{[Na]}{K_mCa}) (1 + \frac{[Ca]}{K_mCa}) + [Na]^3_i [Ca]_o + [Na]^3_i [Ca]_s) (1 + \frac{k_{sat} e^{\sigma VF}}{k})}
$$

(2)

$V_{NCX}$ and $\beta$ were the only free parameters for parameterization, which is described further in section 2.2.

2.1.2 RyR

Two key features of Tg-specific Ca$^{2+}$-handling are enhanced fractional release and markedly reduced SR Ca$^{2+}$ load\textsuperscript{9,14,22}. To represent these characteristics we replaced the Bondarenko RyR gating scheme with the S-B four-state model\textsuperscript{19}, which incorporates a more readily modifiable sensitivity to SR luminal Ca$^{2+}$. The structure of the receptor was unchanged from the original S-B version, but it was parameterized to operate within the relatively low peak [Ca$^{2+}$]$_{ss}$ of the Bondarenko model - supplemental table 1. To more easily retain graded release and prevent prolonged SR Ca$^{2+}$ release events, both of which involve ensemble effects that can only be approximated by a common pool SR release structure, we inherited the gaussian expression for $P_{RyR}$ from Bondarenko et al.\textsuperscript{4}. One outcome of this choice is that we may have limited our ability to replicate the sustained EC coupling involved in the plateau period of type 1 EADs observed in our experiments. Less constrained SR Ca$^{2+}$ release formulations may be required to achieve this type of behavior. Otherwise our final formulation for SR Ca$^{2+}$ release flux remains standard:

$$
I_{rel} = k_s P_o([Ca]_{jss} - [Ca]_{ss}) P_{RyR}
$$

(3)

$$
\frac{dP_{RyR}}{dt} = -0.02 P_{RyR} - 0.1(\frac{I_{Ca_L}}{I_{Ca_L,max}}) e^{(-V/30)}
$$

(4)

2.1.3 SERCA

Our motivation for replacing Bondarenko’s forward-only Hill-type expression for SERCA with the S-B model was to impose thermodynamic constraint on maximal SR Ca$^{2+}$ load. This was because our experiments suggest that increased SR Ca$^{2+}$ release, SR Ca$^{2+}$ reuptake rate, and therefore SR Ca$^{2+}$ load, are key determinants of EAD initiation during $\beta$-adrenergic challenge. In the case of forward-only Hill-type formulations, SERCA-mediated Ca$^{2+}$ re-uptake is balanced by dissipative flux (non-SERCA-mediated SR leak flux) at equilibrium (maximum) SR load. Alternatively, the S-B model imposes a limit to SR load defined by $\Delta G_{ATP} \times \epsilon_{SERCA}$, where $\Delta G_{ATP}$ is the free energy of ATP hydrolysis, and $\epsilon_{SERCA}$ is the efficiency of forward SERCA transport. This approach provides an intrinsic means of constraining maximal SR Ca$^{2+}$ load.
As described above, we exchanged the Bondarenko $I_{Na}$ model for the G-B model\textsuperscript{7}, and specifically fit to Tg mice in Wagner \textit{et al.}\textsuperscript{20}. The scheme and parameterization used here is identical to that of Wagner \textit{et al.}\textsuperscript{20}.

\subsection*{2.2 Fitting:}

\subsubsection*{2.2.1 Calcium Handling}

In addition to the data presented here, we used 2 prior studies to fit SR Ca\textsuperscript{2+} load and EC coupling characteristics of Baseline and Tg models (without Iso). Maier \textit{et al.}\textsuperscript{14} provides data from the CaMK\textsubscript{II}\delta\textsubscript{C}-Tg mice late in HF progression, thus including both acute CaMKII effects and pronounced HF remodeling. Kohlhaas \textit{et al.}\textsuperscript{9}, examined the acute effects of CaMK\textsubscript{II}\delta\textsubscript{C} overexpression on Ca\textsuperscript{2+}-handling mechanisms in rabbit myocytes. We considered the effects that were consistent between these 2 studies, and our current data, to be attributable only to acute CaMKII hyperactivity. These can be summarized as:

1. Reduced SR Ca\textsuperscript{2+} load, and increased diastolic SR Ca\textsuperscript{2+} leak
2. Increased fractional release (FR)
3. Slowed $I_{CaL}$ inactivation, and slightly increased peak $I_{CaL}$

The differences in SR Ca\textsuperscript{2+} handling between Baseline and Tg models were fit to the current data set. While we did not directly measure $I_{CaL}$ in this study, the differences resulting from CaMK\textsubscript{II}\delta\textsubscript{C} overexpression for both prior studies were quantitatively similar, and suggest that the major macroscopic effects of CaMKII at $I_{CaL}$ occur independent of HF remodeling. As such, we fitted the Baseline and Tg models to exhibit differences in $I_{CaL}$ dynamics that were intermediate to the differences observed in those two studies. We did not reduce the Baseline peak $I_{CaL}$ conductance from the Bondarenko model, which was higher than that observed by either Kohlhaas \textit{et al.} or Maier \textit{et al.}, because this would also have required much broader (and poorly constrained) reparameterization of the K\textsuperscript{+} current conductances.

Figure S1 shows the final characteristics of steady-state Ca\textsuperscript{2+} handling in the Baseline and Tg models as well as those of Kohlhaas \textit{et al.}\textsuperscript{9} and Maier \textit{et al.}\textsuperscript{14}. Supplemental table 1 gives the model parameters used to achieve this behavior. Briefly, matching 90:10 \% (SERCA:NCX) flux balance and experimental $\tau_{Ca}$ was achieved in the Baseline model by decreasing $V_{NCX}$ by $\approx 50\%$, setting $\beta = 0.3$, and reducing $V_{SERCA}$ to 200 $\mu$mol/L cytosol/ms. Additionally, setting $K_{mf} = 0.6 \mu$mol/L and $K_{mr} = 3500 \mu$mol/L, gave Baseline diastolic $[Ca]_{i} \approx 120$ nmol/L, and an equilibrium ratio, $[Ca]_{SR} : [Ca]_{i}$, of 5833. This implies 75\% SERCA efficiency, which is similar to experimental observations and the original parameterization of this model\textsuperscript{19}. The free parameters used to simultaneously reduce SR Ca\textsuperscript{2+} load and increase fractional release in the Tg model were: 1) $EC_{50SR}$ (RyR sensitivity to luminal Ca\textsuperscript{2+}), 2) $k_{leak}$ (SR Ca\textsuperscript{2+} leak permeability), 3) $V_{NCX}$, and 4) $V_{SERCA}$. $EC_{50SR}$ and $k_{leak}$ represent demonstrated CaMKII effects at RyR\textsuperscript{1,6,22}, $V_{NCX}$ and $V_{SERCA}$.
remodeling effects that are associated with chronic CaMKII overexpression\textsuperscript{14} and are possibly directly dependent upon CaMKII-mediated transcriptional regulation\textsuperscript{13}. Figure S2 shows that these changes shifted Ca\textsuperscript{2+} flux balance to favor NCX-mediated extrusion, although SERCA-mediated Ca\textsuperscript{2+} reuptake still accounted for \( \sim 80\% \) of cytosolic Ca\textsuperscript{2+} removal.

### 2.2.2 Electrophysiology

As mentioned above, we’ve inherited all Baseline parameters for \( I_{Ca_L} \) from the updated Bondarenko model\textsuperscript{4}. To match the CaMKII-dependent increases in peak current (\( \sim 20\% \)), and fast inactivation time (\( \sim 25\% \)), we applied proportional shifts in \( G_{Ca_L} \) and \( K_{pc,max} \). Initially, we altered the Baseline model to incorporate \( K^+ \) current representations from Li \textit{et al.}\textsuperscript{11}, primarily for their parameterization to data collected at physiologic temperature. However, we found these representations to provide very strong overall repolarization, such that it was not possible to approach our recorded Baseline AP durations within reasonable ranges of the Ca\textsuperscript{2+}-handling parameters. As an alternative we retained the original Bondarenko formulations of all \( K^+ \) currents. We modeled CaMKII-specific effects at \( I_{tof} \) as being solely due to \( I_{tof} \), primarily because the slow component appears only rarely murine cells\textsuperscript{8}. Thus, the \( \sim 35\% \) reduction in total \( I_{tof} \) observed by Wagner \textit{et al.}\textsuperscript{20} was used to parameterize \( G_{tof} \) in our Tg model, and in combination with changes to \( I_{Ca_L} \) this was sufficient to recapitulate our \( APD_{50} \) data - Figure S3. \( G_{K1} \) was also reduced in line with the observations of Wagner \textit{et al.}. This combined with changes to calcium handling allowed a relative difference in \( APD_{90} \) that was similar to the experimental data. However, absolute \( APD_{90} \) was still shorter for both models than observed experimentally, and it is probable that the balance of currents during late repolarization in this model still favor repolarization slightly more strongly than is true for our isolated cells. Finally, the \( K_m \) for intracellular sodium binding of the \( N^+/K^+ \)-ATPase (\( K_{Na} \)) was reduced from 21 to 16.65 mmol/L to maintain stable \( Na^+ \) handling in all three models\textsuperscript{3}.

### 2.2.3 \( \beta \)-adrenergic stimulation

Isoproterenol challenge was simulated with parameter changes representing established effects of \( \beta \)-adrenergic stimulation, and are presented in supplemental table 1. These parameter changes were applied to initial conditions taken from the final steady state of the Tg model, and the effects were allowed to develop over time as the model was paced through 50 beats. Briefly, the major changes were a 2-fold increase in \( G_{Ca_L} \), \textit{7 mV} leftward shifts in \( I_{Ca_L} \) steady-state activation and inactivation, and a 20\% increase in \( G_{Kur} \). Also, as mentioned in section 3.6, because our experimental data suggest that transition to EADs is dependent upon SR Ca\textsuperscript{2+} release, we fixed all other parameters and progressively reduced \( K_{mr} \) and \( K_{mf} \) to slowly introduce PKA-dependent effects upon SR Ca\textsuperscript{2+} reuptake and whole-cell Ca\textsuperscript{2+} load. Importantly, we did not implement any effects specifically of \( \beta \)-adrenergic regulation at \( I_{Na} \). There are two well-established effects of this regulation on macroscopic \( I_{Na} \): (1) leftward shifts in steady activation and inactivation, (2) Increased peak \( I_{Na} \) conductance. Our reasoning for ignoring these effects were, respectively:
(1) the leftward shifts in steady state activation and inactivation are equally likely to be secondary to activation of CaMKII during βAR stimulation, and subsequent CaMKII-dependent phosphorylation of NaV1.5. Because these effects are already incorporated within the Grandi $I_{Na}$ model we avoided additional leftward shifts due to PKA, (2) the increase in peak $I_{Na}$ is thought to arise from trafficking of NaV1.5 to the cell surface, and generally appear after $\sim$ 5 minutes$^2$. Because our experiments generally were complete between 2-3 minutes after initiating βAR stimulation, we feel is it unlikely this mechanism could have contributed to the observed outcomes.

### 2.2.4 Model Implementation and Availability

All models were implemented in Matlab (Release 2013a), and solved with the adaptive implicit ODE solver ODE15s. Pacing current injection was simulated by 5 ms square pulses of 8.0 A/F amplitude, and was carried by K$^+$. All model code and data for this study are available at the CMRG website:

**UCSD Cardiac Mechanics Research Group**
References


3 Supplemental figures

Figure S1
Figure S2
Figure S3

![Bar charts showing APD50 and APD90 for different conditions.](image-url)
Figure S5

A (a) 10 μM Ranolazine

B (a) + 1 μM TTX

(b) + 10 μM Ranolazine

(b) + 1 μM TTX
Figure S7

A

B

C

IC1 ↔ IC2 ↔ IF ↔ I1 ↔ I2

C1 ↔ C2 ↔ C3 ↔ O

LC1 ↔ LC2 ↔ LC3 ↔ LO

C1

C2

C3

O

LO

IC1   IC2   IF   I1   I2

LC1   LC2   LC3   LO

0 mV

40 mV

100 ms

20 mV

50 ms

-40 mV

0.002

0.0

C1

C2

C3

O
Figure S8
Figure S9

A.

$E_m$

$[\text{Ca}]_{i}$

$\frac{F_{340}/F_{380}}{0.2}$

1 second

B.

$E_m$

$[\text{Ca}]_{i}$

$\frac{F_{340}/F_{380}}{0.04}$

1 second
4 Supplemental figure legends

Table 1: Model Parameters. * Indicates parameters listed for the Baseline model that have been altered from the Bondarenko model. * indicates Tg specific effects. ** indicates Isoproterenol specific effects. *** indicates effects that are the combination of Tg- and Iso-specific effects. The parameters $\alpha_{left}$ and $\beta_{left}$, are part of the functions for $\alpha$ and $\beta$ in the Bondarenko 2010 model\(^4\). These were (1) $\alpha = 0.4e^{(V+\alpha_{left})/15.0}$, and (2) $\beta = 0.13e^{-(V+\beta_{left})/18.0}$.  

Figure S1: Comparison of steady-state model behavior with data from Kohlhaas et al.\(^9\), Maier et al.\(^14\), and herein. Tg data are normalized to Baseline for Fractional release (FR), SR Ca\(^{2+}\) load, diastolic Ca\(^{2+}\), and $\Delta$[Ca\(^{2+}\)]\(_i\), to allow comparison with fluorescence based measures of SR Ca\(^{2+}\) load\(^9,14\), or our ratiometric Ca\(^{2+}\)-handling data. Absolute SR Ca\(^{2+}\) load for the Baseline model was 93 $\mu$mol/L cytosol. NC: data not collected herein.

Figure S2: Pathways of cytosolic Ca\(^{2+}\) removal at steady state in the Baseline, Tg, and Tg + Iso models. Time-courses are the cumulative integrals of net fluxes due to SERCA reuptake (red), and extrusion via NCX (black), or PMCA (blue).

Figure S3: Steady-state action potential duration in the Baseline and Tg models, and our experimental data.

Figure S4: Global cardiac phenotype: A. Kaplan-Meier curve showing onset of mortality in Tg mice. B. Heart:body mass (left) was increased in Tg mice, although both absolute lung mass (middle), and lung:body mass (right) were unchanged. C. Echocardiography indicates mild ventricular dilation and contractile dysfunction in pre-HF Tg mice; Tg (n = 10) vs. WT (n = 15) animals. D. Anesthetized electrocardiography at baseline and during intravenous isoproterenol (Iso, 25 $\mu$g/kg) challenge for Tg (n = 10) and WT (n = 14) animals. E. IP injection of epinephrine (2 mg/kg) and caffeine (120 mg/kg) elicited VT in Tg animals. F. Representative episode of non-sustained VT in a Tg animal. Heart rate increased from $\sim$390 beats/min at baseline to $\sim$430 beats/min shortly after epi/caff,
and then ~600 beats/min during VT. All panels: ** $p < 0.001$, * $p < 0.05$. Group data are mean ± SEM.

**Figure S5:** Complete traces for the experimental cell in Figure 7 directly after application of 10 µM Ranolazine (A), and addition of 1 µM TTX to the bath (B). Right side of both panels shows the change in AP morphology resulting from the antagonist - selected APs are boxed at left. Ranolazine curtailed to the prolonged EAD phenotype but did not prevent the dynamics underlying EAD initiation. Conversely TTX at 1 µM or greater did eliminate EAD initiation.

**Figure S6:** A. Comparison of EAD prevention by TTX blockade in experiments and simulations. A. Imposing an acute 50% reduction of peak $I_{Na}$ conductance ($G_{Na} = 6.5 mS.\mu F^{-1}$) to simulate rapid TTX application, abolishes EADs (indicated by asterisks *) in the Tg + Iso model. B. Similar rapid application of TTX abolishes EADs in an experimental cell.

**Figure S7:** Reactivation through canonical recovery from $I_{Na}$ inactivation. A. The states of the Markovian $I_{Na}$ model. B. Simulated action potential including an EAD as in Figure 6B(c). C. Magnified region of $I_{Na}$ reactivation from B, including $E_m$ (top) and state occupancies (bottom) for the canonical closed states (C1-C3), and the open state (O). Non-equilibrium reactivation is fueled by these canonical closed states.

**Figure S8:** The Tg $I_{Na}$ model exhibits reduced non-equilibrium reactivation during Iso-challenge compared to the WT $I_{Na}$ model. Both simulations are performed in the transgenic cell model, but with either the WT (right) or Tg (left) $I_{Na}$ representations. The WT $I_{Na}$ model does not include the leftward shifts in steady-state activation and inactivation, enhanced late current, or sowed recovery from inactivation associated with CaMKII overexpression. The result is that the WT channel permits greater non-equilibrium reactivation, thus suggesting that the $I_{Na}$-dependent EADs we have observed do not rely specifically on CaMKII regulation of the channel.
Figure S9: A. Representative examples of sustained events. A. Triggered sustained events were generally non-terminating EADs, and therefore were treated as a special case of EAD. B. Representative example of the infrequent spontaneous sustained events.