Targeting the late component of the cardiac L-type Ca\(^{2+}\) current to suppress early afterdepolarizations

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Early afterdepolarizations (EADs) associated with prolongation of the cardiac action potential (AP) can create heterogeneity of repolarization and premature extrasystoles, triggering focal and reentrant arrhythmias. Because the L-type Ca\(^{2+}\) current (ICa,L) plays a key role in both AP prolongation and EAD formation, L-type Ca\(^{2+}\) channels (LTCCs) represent a promising therapeutic target to normalize AP duration (APD) and suppress EADs and their arrhythmogenic consequences. We used the dynamic-clamp technique to systematically explore how the biophysical properties of LTCCs could be modified to normalize APD and suppress EADs without impairing excitation–contraction coupling. Isolated rabbit ventricular myocytes were first exposed to H\(_2\)O\(_2\) or moderate hypokalemia to induce EADs, after which their endogenous ICa,L was replaced by a virtual ICa,L with tunable parameters, in dynamic-clamp mode. We probed the sensitivity of EADs to changes in the (a) amplitude of the noninactivating pedestal current; (b) slope of voltage-dependent activation; (c) slope of voltage-dependent inactivation; (d) time constant of voltage-dependent activation; and (e) time constant of voltage-dependent inactivation. We found that reducing the amplitude of the noninactivating pedestal component of ICa,L effectively suppressed both H\(_2\)O\(_2\)- and hypokalemia-induced EADs and restored APD. These results, together with our previous work, demonstrate the potential of this hybrid experimental–computational approach to guide drug discovery or gene therapy strategies by identifying and targeting selective properties of LTCC.

INTRODUCTION

Early afterdepolarizations (EADs) are arrhythmogenic membrane potential oscillations that occur before repolarization of the cardiac action potential (AP) is complete. Although the mechanistic link between EADs in single cells and triggered arrhythmias in the heart is still a subject of intense investigation (Wit and Rosen, 1983; Rosen, 1988; Antzelevitch and Sicouri, 1994; El-Sherif et al., 1996; Yan et al., 2001; Xie et al., 2010; Yang et al., 2010), it is firmly established that EADs are capable of triggering fatal arrhythmias such as polymorphic ventricular tachycardia, torsade de pointes, and ventricular fibrillation (Yan et al., 2001; Choi et al., 2002; Wu et al., 2002; Antzelevitch, 2007; Bapat et al., 2012), which often exhibit a mixture of focal and reentrant mechanisms (Asano et al., 1997; Sato et al., 2009; Weiss et al., 2010; Chang et al., 2012). Not only are EADs capable of generating triggered activity to produce focal arrhythmias, but they are also associated with a prolonged AP duration (APD). Thus, they can markedly increase dispersion of refractoriness in tissue, predisposing to initiation of reentry (Sato et al., 2009; Weiss et al., 2010; Chang et al., 2012).

The onset of EADs (i.e., the reversal of the normal repolarization phase of the AP) occurs within a range of the membrane potentials where the steady-state activation and inactivation curves of the L-type Ca\(^{2+}\) current (ICa,L) overlap, known as the ICa,L window current region (January et al., 1988). Within this membrane potential range, ICa,L reactivation plays a key role in reversing the normal repolarization phase during EAD formation (January et al., 1988). Although other ionic currents also participate in EAD formation, a regenerative inward current such as ICa,L is required for EADs to propagate in the tissue (Zeng and Rudy, 1995; Chang et al., 2012). Using the dynamic-clamp technique (Dorval et al., 2001) in isolated rabbit ventricular myocytes, we recently demonstrated that EADs are highly sensitive to subtle changes in the half-activation or half-inactivation potentials of ICa,L, suggesting that a reduction of the ICa,L window current may represent an effective maneuver to

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Abbreviations used in this paper: AP, action potential; APD, AP duration; EAD, early afterdepolarization; ICa,L, L-type Ca\(^{2+}\) current; VDI, voltage-dependent inactivation.

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suppress EADs and normalize AP without blocking the early peak I_{Ca,L}, required to maintain a normal excitation-contraction coupling (Madhvani et al., 2011). The dynamic clamp is a powerful technique that allows one to introduce a model conductance, such as I_{Ca,L}, with programmable properties into a cell in real time to study its effects on AP characteristics (Fig. 1 E). The proof-of-concept provided by our initial study (Madhvani et al., 2011) prompted us to perform a comprehensive analysis of biophysical parameters influencing the time- and voltage-dependent properties of the window (late) I_{Ca,L} to identify whether additional parameters could be modified to suppress EAD formation and normalize AP. Accordingly, we systematically investigated the slopes of the voltage dependence of activation and inactivation, the noninactivating (or very slowly inactivating) late pedestal current, and the time constants of activation and inactivation, which shape the I_{Ca,L} window current (Fig. 1, A and C) during the cardiac AP.

The results demonstrate that of all the I_{Ca,L} biophysical parameters explored in this and previous work (Madhvani et al., 2011), three stand out as highly effective targets both to suppress EAD formation and normalize AP to reduce dispersion of repolarization: the half-activation and half-inactivation potentials and the noninactivating pedestal current. Collectively, these findings provide a drug discovery target to search for new antiarrhythmic agents to suppress EAD-mediated arrhythmias. Moreover, this study recapitulates a novel hybrid experimental–computational approach incorporating the dynamic-clamp technique to predict how subtle alterations in biophysical properties of ionic currents such as I_{Ca,L} affect cardiac electrophysiology and arrhythmogenic phenomena.

MATERIALS AND METHODS

Ethical approval
All animal-handling protocols were approved by the UCLA Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Electrophysiology
3–4-mo-old New Zealand male rabbits were euthanized by an intravenous injection of 1,000 U heparin sulfate and 100 mg/kg sodium pentobarbital; adequacy of anesthesia was confirmed by the lack of pedal withdrawal reflex, corneal reflex, and motor response to pain stimuli. Ventricular myocytes were dissociated using a retrograde Langendorff perfusion system as described previously (Chen et al., 2003), and washed and bathed in Tyrode’s solution containing (mM): 136 NaCl, 5.4 KCl, 1 MgCl₂, 0.33 NaH₂PO₄, 1.8 CaCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4. The intracellular solution contained (mM): 110 KCl, 30 KCl, 5 NaCl, 10 HEPES, 0–0.1 EGTA, 5 MgATP, 5 creatine phosphate, and 0–0.05 cAMP adjusted to pH 7.2. EADs were induced by perfusing 600 µM H₂O₂ or reducing the external [K⁺] from 5.4 to 2.7 mM. All chemicals were purchased from Sigma-Aldrich. All electrophysiological recordings were performed using an Axopatch 200B amplifier (Axon Instruments) in current-clamp mode at ~34–36°C using 1–2-MΩ borosilicate pipettes (Warner Instruments). Data were acquired and analyzed using custom-made software (G-Patch; Analysis).

Dynamic clamp
Under dynamic-clamp mode, a virtual I_{Ca,L} with the properties of the native I_{Ca,L} is injected into the myocytes. To predict I_{Ca,L} and its Ca²⁺-dependent inactivation, our ventricular myocyte model also computes intracellular Ca²⁺ cycling. To predict the spatiotemporal distribution of intracellular calcium, average [Ca²⁺] was computed in four different cellular compartments, namely the “submembrane space” in proximity of the sarcolemma (Cₛ), the “bulk myoplasm,” the “junctional SR,” and “network SR,” as described previously (Shiferaw et al., 2003; Mahajan et al., 2008; Madhvani et al., 2011). The main Ca²⁺-regulated ionic conductance was also included in the model, i.e., the fast sodium current (I_{Na}), the Na⁺/K⁺ pump current (I_{Na,K}), the Na⁺/Ca²⁺ exchange current (I_{Ncx}), and the Ca²⁺-dependent slow component of the delayed rectifier potassium channel (I_{Ks}). Calcium-modulated currents sense the [Ca²⁺] at submembrane space (Cₛ), which is higher than the global [Ca²⁺] (C) (Weber et al., 2002). The average Cₛ is used to calculate Ca²⁺-dependent inactivation in the I_{Ca,L} formulation, whereas Cₛ was acquired during the course of the experiments to predict the amplitude and shape of the Ca transient.

In brief, the Ca²⁺ flux into the cell caused by I_{Ca,L} is given by:

\[
J_{Ca} = g_{Ca} P_{oi} I_{Ca,L}
\]

(1)

\[
k_{Ca} = \frac{4 P_{Ca} V_o F^2 \frac{Ca^{2+}}{RT}}{e^{\frac{V_o}{RT}} - 1} - 0.344 \frac{\left[Ca^{2+}\right]}{1 - e^{\frac{-V_o}{RT}}}
\]

(2)

where Cₛ is the submembrane Ca²⁺ concentration in units of millimolar, \( P_{oi} \) (0.00054 cm/s) is the Ca²⁺ channel permeability, \( V_o \) is the membrane potential, \( F \) is the Faraday constant, and \( T \) is temperature. \( P_{oi} \) was formulated as:

\[
P_o = d \cdot f \cdot q,
\]

(3)

where \( d \) is the voltage-dependent activation gate, \( f \) is the VDI gate, and \( q \) is the Ca²⁺-dependent inactivation gate. The steady states of these gating variables as functions of the membrane potential (\( V_m \)) were formulated as follows:

\[
d_a = \frac{1}{1 + \exp\left(-\left(V_m - d_{half}\right) / d_{slope}\right)}
\]

(4)

\[
f_a = \frac{1 - f_{dest}}{1 + \exp\left(-\left(V_m - f_{half}\right) / f_{slope}\right)} + f_{dest}
\]

(5)

\[
q_a = \frac{1}{1.0 + \left(\frac{C_s}{c_{st}}\right)\gamma}
\]

(6)

\[
\tau_j = \frac{d_j \left(1 - \exp\left(-\left(V_m - d_{half} + a_j\right)\right)\right)}{a_j \left(\exp\left(-\left(V_m - d_{half} + a_j\right)\right) + a_j \left(V_m - d_{half} + a_j\right)\right)}
\]

(7)

\[
\tau_f = \frac{1}{b_i \exp\left(-b_i \left(V_m - \left(f_{half} + b_i\right)\right)^2\right) + b_i}
\]

(8)
where $d_{\text{half}}$ and $f_{\text{half}}$ are the potentials at half-maximum of activation and inactivation, respectively; $p_{\text{dest}}$ is the noninactivating pedestal of the inactivation gate; and $dslope/f\text{slope}$ are the steepness of the voltage dependence of activation and inactivation, respectively. These two parameters are called slope factors (k) in this study, and are related to effective charge (z) by $RT/z$, where R is the gas constant and T is temperature. $C_{i}$ is the submembrane ($Ca^{2+}$); $C_{v}$ is the affinity for $Ca^{2+}$ of the inactivation gate; $d_{a}$ and $d_{a}$ are the time constants of the $d$ gate and the $f$ gate, respectively; and $a_{b}$, $b_{b}$, $b_{b}$, and $b_{d}$ are additional factors used for fitting. The control parameters in the $I_{\text{Ca,L}}$ formulation were determined by fitting formulated current to experimental nifedipine-sensitive $I_{\text{Ca,L}}$ records (Madhvani et al., 2011) using Berkeley Madonna and then implemented for dynamic clamp in RTXI (http://www.rtxi.org; Lin et al., 2010). In each experiment, as soon as the whole-cell configuration was obtained, the cell capacitance of the myocyte was measured (usually ranging within 100–150 pF) and entered as one of the parameters of the dynamic-clamp model to scale the computed $I_{\text{Ca,L}}$ accordingly to the cell size. The sampling/computation frequency was 10 kHz.

In some experiments, 10% of the computed slow component of the delayed rectifier $K^+$ current ($I_{K}$) was injected together with $I_{\text{Ca,L}}$. $I_{K}$ was modeled as in Mahajan et al. (2008).

**Data analysis**

APD at 90% repolarization ($\text{APD}_{90}$) was calculated using custom-made software, whereas EAD amplitude was calculated manually by measuring the difference in $V_{m}$ from the local minimum where $dV/dt$ is 0 to the peak of the EAD where $dV/dt$ is also 0. In APs that displayed multiple EADs, only the EAD with the largest voltage excursion was included in the analysis. EAD occurrence is reported as the percentage of APs that displayed at least one EAD. Error bars show the SEM.

**Computer simulations**

Single-cell AP simulations were produced using the rabbit ventricular myocyte AP model developed by Mahajan et al. (2008), with some modifications. For details, see the supplemental text.

**Online supplemental material**

Online supplemental figures show the effects of varying the slope (k) of the steady-state inactivation curve (Fig. S1) and the predicted $Ca_{\text{transient}}$ transient before and after reduction of $I_{\text{Ca,L}}$ pedestal (Fig. S2) under the dynamic clamp. Fig. S3 demonstrates that the reduction of $I_{\text{Ca,L}}$ noninactivating component effectively suppressed EADs under hypokalemia condition. Fig. S4 shows results from computer simulation using a model of rabbit myocytes with properties of M, endo, and epi cell layers. Table S1 reports the mean values for EAD occurrence and $\text{APD}_{90}$ for different $I_{\text{Ca,L}}$ activation and inactivation time constants. Table S2 shows the biophysical parameters of $I_{\text{Ca,L}}$ in the dynamic-clamp model for control, $H_{2}O_{2}$, and hypokalemia conditions. Tables S3 and S4 provide parameter values for maximal ionic conductances used in the computer simulation using a model of rabbit AP with properties of epi, endo, and M cell layers. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201411288/DC1.

**RESULTS**

Sensitivity of EAD occurrence to the noninactivating component of $I_{\text{Ca,L}}$

In our previous study (Madhvani et al., 2011), we demonstrated that modest shifts (<5 mV) in the half-activation and half-inactivation potentials of $I_{\text{Ca,L}}$, which reduce the overlap between steady-state activation and inactivation curves (i.e., the window current region; Fig. 1 A), potently suppressed EADs, without adversely altering APD or the computed intracellular $Ca_{\text{transient}}$ transient. However, other parameters also affect the $I_{\text{Ca,L}}$ window current and its ability to contribute to EAD formation. Using dynamic clamp, we have explored the extent to which the other properties affecting activation and inactivation contribute to EAD formation.

To induce an EAD regimen, myocytes were paced at 5-s cycle length under current-clamp mode and super fused with 600 µM $H_{2}O_{2}$ until EADs appeared consistently. The native $I_{\text{Ca,L}}$ was then blocked with 20 µM nifedipine, which eliminated EADs and markedly shortened the APD (Fig. 1 D). Next, the dynamic clamp was

![Figure 1](http://www.jgp.org/cgi/content/full/jgp.201411288/DC1)
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engaged to replace the native \( I_{Ca,L} \) (blocked by nifedipine) by injecting, in real time, a \( I_{Ca,L} \) computed from Eqs. 1–8, causing EADs to reappear (Fig. 1 E). The membrane potential of the myocyte was continuously sampled and fed into the dynamic-clamp model, creating a bidirectional relationship between the cell and the model, in real time (Fig. 1 E). The injected \( I_{Ca,L} \) had the properties of the native \( I_{Ca,L} \) in the presence of 600 \( \mu \)M \( H_2O_2 \) (Madhvani et al., 2011). Importantly, all dynamic-clamp experiments were performed in the continuous presence of \( H_2O_2 \) or hypokalemia, maintaining a pathological state that resulted in EAD formation.

The inactivation of \( I_{Ca,L} \) caused by voltage- and \( Ca^{2+} \)-dependent mechanisms (voltage-dependent inactivation, VDI, and \( Ca^{2+} \)-dependent inactivation, CDI, respectively) (Catterall, 2000) is incomplete during the time course of an AP. This noninactivating component was previously found to be elevated (from \( \sim 3 \) to \( \sim 10\% \) of the peak current) in myocytes exposed to an EAD-promoting regimen (\( H_2O_2 \)) (Madhvani et al., 2011), suggesting that this residual current contributes to EAD formation by effectively increasing \( I_{Ca,L} \) window region (Fig. 1, A and B). Motivated by these pieces of evidence, we directly probed EAD sensitivity to changes in the amplitude \( I_{Ca,L} \) noninactivating component (pedestal) (Rose et al., 1992; Qu and Chung, 2012). As demonstrated in Fig. 2, after inducing EADs in a myocyte by \( H_2O_2 \) superfusion (Fig. 2 B), the EADs were abolished by nifedipine (Fig. 2 C) and reconstituted by injecting a virtual \( I_{Ca,L} \) (Fig. 2 D). Under these conditions, APs were markedly prolonged, displaying a consistent EAD regimen, although with oscillations that tended to be smaller in amplitude than the ones typically observed experimentally (Fig. 2). Because nifedipine abolishes the \( Ca_\text{transient} \), which is important for activating \( I_{Ks} \) (Tohse, 1990; Nitta et al., 1994), we compensated by adding 10% of the computed \( I_{Ks} \) to the injected current. This maneuver reduced the voltage plateau into a range promoting greater \( I_{Ca,L} \) reactivation and larger EAD oscillations (Fig. 2 E), more closely resembling \( H_2O_2 \)-induced EADs before nifedipine blockade. The reduction of the \( I_{Ca,L} \) pedestal from 10 to 4% of the peak completely suppressed EAD occurrence and shortened the APD\(_{90}\) from 1,090 ± 80 ms to 197 ± 3 ms (Figs. 2 F and 3) whether or not EAD amplitude was increased by adding \( I_{Ks} \). Importantly, the reduction of the \( I_{Ca,L} \) pedestal suppresses the EAD regimen without attenuating the amplitude of the predicted \( Ca_\text{transient} \) (Fig. S2).

These results indicate that the reduction of the noninactivating \( I_{Ca,L} \) pedestal current can be an effective therapeutic strategy to suppress EADs.

Sensitivity of EADs to the steepness of steady-state activation and inactivation curves

Another set of parameters affecting the \( I_{Ca,L} \) window region are the steepness of the voltage dependence of activation and inactivation. However, modification of either parameter did not completely suppress EADs, nor did it restore normal APD, in contrast to the consequences of noninactivating pedestal current reduction. After reconstitution of the EADs with dynamic clamp in the presence of \( H_2O_2 \) in the superfusate, we first examined the effects of altering the slope factor (\( k \)) of the voltage dependence of activation from 4 mV (effective valence, \( z = 5.7 e^0 \)) to 1 mV (\( z = 25.6 e^0 \)) or 8 mV (\( z = 3.2 e^0 \)) to increase or decrease, respectively, the steepness of

Figure 2. Potent suppression of EAD by reduction of the noninactivating (pedestal) \( I_{Ca,L} \) component. (A) APs recorded from rabbit ventricular myocytes at a pacing cycle length of 5 s at 35–37°C in control conditions. (B) Perfusion of 600 \( \mu \)M \( H_2O_2 \) to the bath solution-induced EADs within 5–10 min. (C) The addition of 20 \( \mu \)M nifedipine blocked the native \( I_{Ca,L} \) shortened APD, and abolished EADs. (D) Under dynamic clamp and in the presence of \( H_2O_2 \), we evaluated the injection of virtual \( I_{Ca,L} \) or (E) the combination of \( I_{Ca,L} \) plus 10% of the computed \( I_{Ks} \). Note that both conditions regenerate the EAD regimen, although the amplitude of the oscillations tended to be larger with the addition of 10% \( I_{Ks} \). (F) Varying a single \( I_{Ca,L} \) parameter, the noninactivating (pedestal) component completely abolished EADs, despite the presence of \( H_2O_2 \).
Sensitivity of EADs to the kinetics of ICa,L activation and inactivation

We next studied the effects of altering the time constants of ICa,L activation and inactivation on EAD formation during oxidative stress. Overall, we found that changes in the rates of ICa,L activation or inactivation by up to 10-fold had limited efficacy at suppressing EADs induced by H2O2 (Figs. 5 and 6).

Specifically, in the presence of H2O2, slowing ICa,L activation by twofold (τa × 2) maintained an average APD90 of 1,550 ± 130 ms, and EAD occurrence remained high at 91 ± 4% (Table S1). A 10-fold slowing of ICa,L activation (τa × 10) prolonged APD90 from 1,376 ± 200 ms to 2,711 ± 548 ms, whereas EAD occurrence increased from 76 ± 8% to 100% of APs. Conversely, increasing the rate of ICa,L activation by twofold (τa × 0.5; Table S1) or by 10-fold (τa × 0.1) had only a modest impact on both APD90 (1,122 ± 126 ms or 1,104 ± 165 ms) and EAD occurrence (79 ± 15% or 77 ± 10%), respectively (Fig. 5 and Table S1).

When the rate of VDI in the dynamic-clamp model was increased by twofold (τd × 0.5), neither APD90 (from the voltage dependence of activation (Fig. 4 A). We found that, at greater steepness, EAD amplitudes decreased from 5.6 ± 0.7 mV to 3.1 ± 1 mV (for k = 2 mV, z = 12.8 e0) or 2.6 ± 0.8 mV (k = 1 mV, z = 25.6 e0) (Fig. 4, B and C). Also, for the steepest slope (k = 1 mV, z = 25.6 e0), the APD became prolonged such that, in some cases, the AP failed to repolarize before the next pacing stimulus (APD > 5 s) (Fig. 4 B). Conversely, when the effective charge of the activation curve was reduced from k = 4 mV (z = 6.4 e0) to k = 6 mV (z = 4.3 e0), EAD amplitude increased from 5.6 ± 0.7 mV to 12.3 ± 1.4 mV (Fig. 4, B and C). This effect was even more pronounced for k = 8 mV (z = 3.2 e0), such that the mean EAD amplitude increased to 21.4 ± 2 mV (Fig. 4, B and C), although no significant changes in percentage of APs with EADs were observed (Fig. 4 D), and APD90 remained prolonged for these maneuvers (Fig. 4 E).

We also investigated the effects of changing the slope of the voltage dependence of inactivation (Fig. S1 A) by increasing or decreasing the effective charge to k = 1 mV (z = 25.6 e0) or k = 8 mV (z = 3.2 e0), respectively. We did not observe a significant difference in either EAD amplitude or in percentage of APs with EADs (Fig. S1, C and D). APD90 prolonged from 1,104 ± 319 ms to 3,140 ± 771 ms (Fig. S1, B and E) as the steepness of the inactivation curve was reduced from k = 4 mV (z = 6.4 e0) to k = 8 mV (z = 3.2 e0) (Fig. S1 A).

Figure 3. A reduction in the noninactivating (pedestal) ICa,L potently suppresses EADs and restores APD. (A and B) Enlarged view of the steady-state activation and inactivation curves of ICa,L shows changes made to the noninactivating component (pedestal). Under dynamic clamp and in the presence of H2O2, we evaluated the effect of lowering the noninactivating pedestal from 10% (A) to 4% of the peak current (B). (C) The proportion of APs displaying EADs under two different pedestal amplitudes. (D) APD90 under two different pedestal amplitudes. Note that lowering the pedestal current to 4% eliminated EADs and restored a normal APD when either ICa,L only (circles) or ICa,L plus 10% IKs (squares) was injected. The means for all experiments are plotted as open rectangles (n = 6 from six rabbits). Error bars indicate SEM.

Figure 4. EAD amplitude is sensitive to changes in the slope of the steady-state voltage dependence of activation of ICa,L. (A) Under dynamic clamp and in the presence of H2O2, we evaluated the effects of varying the slope factor (k) of the steady-state activation curve. Steady-state inactivation properties were unaltered, whereas the slope of the steady-state activation curve was varied under dynamic clamp from k = 1 mV to k = 8 mV. The set of curves in the center, k = 4 mV, corresponds to the native ICa,L modified by H2O2. (B) Representative APs obtained for each k value studied, displaying EADs. For each value of k tested, the mean EAD amplitude (C), EAD occurrence (D), and APD90 (E) are shown. Data from individual cells are shown as closed circles, and the means for all experiments are plotted as an open rectangles (n = 5–6 cells from five to six rabbits). Error bars indicate SEM. *, instances when the AP failed to repolarize before the next pacing stimulus are reported as RF (repolarization failure). Note that EAD amplitude grows as k is increased (slope becomes shallower).

Figure 4.

A reduction in the noninactivating (pedestal) ICa,L potently suppresses EADs and restores APD. (A and B) Enlarged view of the steady-state activation and inactivation curves of ICa,L shows changes made to the noninactivating component (pedestal). Under dynamic clamp and in the presence of H2O2, we evaluated the effect of lowering the noninactivating pedestal from 10% (A) to 4% of the peak current (B). (C) The proportion of APs displaying EADs under two different pedestal amplitudes. (D) APD90 under two different pedestal amplitudes. Note that lowering the pedestal current to 4% eliminated EADs and restored a normal APD when either ICa,L only (circles) or ICa,L plus 10% IKs (squares) was injected. The means for all experiments are plotted as open rectangles (n = 6 from six rabbits). Error bars indicate SEM.
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Afterdepolarizations and \( ICa,L \) pedestal is effective at suppressing EADs caused by a mechanism other than oxidative stress, we also examined its potency at suppressing hypokalemia-induced EADs. As shown previously (Sato et al., 2010), lowering extracellular \([K^+]\) from 5.4 to 2.7 mM readily induced EADs within 5–10 min. Using the same dynamic-clamp approach, the endogenous nifedipine-sensitive current was replaced by a virtual \( ICa,L \), the parameters of which were modified to simulate the effects of hypokalemia on the native \( ICa,L \) (Table S2). The virtual \( ICa,L \) restored EADs were greatly suppressed (\( n = 3 \)) or completely abolished (\( n = 2 \)) by reducing the pedestal component of the virtual \( ICa,L \) from 5% to ≤0.5% (Fig. S3, C and D).

Effects of noninactivating pedestal of \( ICa,L \) on simulated EADs in epicardial, endocardial, and M cell types

Across the ventricular wall, there are regional differences in the ionic basis underlying ventricular APs (Antzelevitch et al., 1991; Antzelevitch and Sicouri, 1994). To explore how these regional differences affect the ability of a therapeutic reduction in the \( ICa,L \) pedestal to suppress EADs, we modified the rabbit ventricular AP model to simulate epicardial, endocardial, and M cell

Figure 5. Time constant of \( ICa,L \) activation has a limited effect on \( H_2O_2 \)-induced EADs. (A) \( ICa,L \) model outputs in response to depolarizing pulses from \(-80 \text{ to } 10 \text{ mV}\) using three different time constants of activation (\( \tau_d \)). (B) Representative APs recorded under dynamic-clamp conditions from myocytes in which the endogenous \( ICa,L \) was replaced with a virtual \( ICa,L \) with modified activation time constants. The injected virtual \( ICa,L \) in dynamic clamp is shown in gray under each AP trace. Increasing or decreasing \( ICa,L \) activation rate by a factor of 10 did not significantly shorten APD or suppress EADs. For each value of \( \tau_d \) tested, EAD occurrence (C) and APD\(_{90}\) (D) are shown. Data from individual cells are shown as closed circles, and the means for all experiments are plotted as open rectangles (\( n = 7 \) from five to six rabbits). Note that in all cases, APD\(_{90}\) remained prolonged (≥500 ms). Error bars indicate SEM. *, instances when the AP failed to repolarize before the next pacing stimulus are reported as RF (repolarization failure).

Figure 6. \( ICa,L \) time constant of inactivation has limited efficacy for suppressing EADs induced by \( H_2O_2 \). (A) \( ICa,L \) model outputs in response to depolarizing pulses from \(-80 \text{ to } 10 \text{ mV}\) using four different time constants of inactivation (\( \tau_i \)). (B) Representative APs recorded under dynamic-clamp conditions from myocytes in which the endogenous \( ICa,L \) was replaced with a virtual \( ICa,L \) with modified inactivation time constants. The injected virtual \( ICa,L \) in dynamic clamp is shown in gray under each AP trace. For each value of \( \tau_i \) tested, EAD occurrence (C) and APD\(_{90}\) (D) are shown. Data from individual cells are shown as closed circles, and the means for all experiments are plotted as open rectangles (\( n = 4–7 \) from three to five rabbits). A limited favorable effect on EAD occurrence was observed for decreasing \( \tau_i \) by a factor of 2; however, on average, APs remained prolonged (≥500 ms). Error bars indicate SEM. *, instances when the AP failed to repolarize before the next pacing stimulus are reported as RF (repolarization failure).
layer APs, respectively. When paced at a 5-s cycle length under control conditions, with a 3% \( I_{\text{Ca,L}} \) pedestal current (Fig. S4 A), APD\(_{90} \) averaged 229, 258, and 268 ms for the epicardial, endocardial, and M cells, respectively. We then simulated the effects of \( \text{H}_2\text{O}_2 \) as described above, including increasing the \( I_{\text{Ca,L}} \) pedestal current to 6%. The APD\(_{90} \) prolonged to 540, 504, and 811 ms, respectively, and EADs appeared in all three cell types (Fig. S4 B). When the \( I_{\text{Ca,L}} \) pedestal current was reduced to 0%, while maintaining all other \( \text{H}_2\text{O}_2 \) effects, APD\(_{90} \) shortened to 273, 293, and 309 ms, respectively, and EADs disappeared (Fig. S4 C). Thus, suppressing the \( I_{\text{Ca,L}} \) pedestal current was effective at eliminating EADs regardless of transmural AP heterogeneity.

**DISCUSSION**

Although many ionic currents can contribute to EAD formation, reactivation of \( I_{\text{Ca,L}} \) plays a central role in providing a regenerative inward current required for EADs to propagate, thereby causing triggered activity in multicellular tissue (January et al., 1988; January and Riddle, 1989). The main finding of the present study is that, even though \( \text{H}_2\text{O}_2 \) is likely to promote EADs by affecting multiple ionic currents such as late \( I_{\text{Na}} \) (Song et al., 2006), the modification of \( I_{\text{Ca,L}} \) is sufficient to prevent EADs even in the presence of \( \text{H}_2\text{O}_2 \). Furthermore, this intervention does not diminish the amplitude of the predicted \( \text{Ca}^{2+} \) transient (Fig. S2) and therefore is expected to maintain normal cell contractility. Conventional \( \text{Ca}^{2+} \) channel blockers such as nifedipine, which indiscriminately block both peak and window \( I_{\text{Ca,L}} \), are highly effective at suppressing EADs (e.g., Fig. 1 D). However, by blocking peak \( I_{\text{Ca,L}} \), these drugs also potently suppress excitation–contraction coupling, precluding clinical usefulness for EAD suppression.

In this study, we applied the dynamic-clamp technique to systematically evaluate the hypothesis that EADs...
can be suppressed by selectively targeting the biophysical properties regulating the \( I_{\text{Ca,L}} \) window current. Fig. 7 summarizes the effects of the various parameter changes on both APD and EAD occurrence, illustrating that three parameter modifications (depolarizing shift of the half-activation potential, hyperpolarizing shift of the half-inactivation potential, and reduction of the pedestal current) both effectively suppress EADs and restore APD toward a normal value. From these findings, we predict that the ideal \( \text{Ca}^{2+} \) channel agent for suppressing EADs can be suppressed by selectively targeting the biophysical properties regulating the \( I_{\text{Ca,L}} \) window current. As the membrane potential exceeds the voltage for peak \( I_{\text{Ca,L}} \), the diminishing amplitude of \( I_{\text{Ca,L}} \) and voltage-dependent growth of \( I_{Ks} \) force the repolarization phase of the EAD, with this tug-of-war generating successive oscillations. Our results show that EAD amplitude is a function of the slope of the voltage dependence of \( I_{\text{Ca,L}} \) activation (Fig. 4), consistent with the dynamical theory that membrane oscillations (EADs) occur because of instability of the \( I_{\text{Ca,L}} \) in the window current voltage range via a Hopf bifurcation (Tran et al., 2009; Qu et al., 2013). EAD formation is more susceptible to interventions that limit the maximum steady-state amplitude that the late \( I_{\text{Ca,L}} \) can achieve during repolarization (shifts in half-activation and half-inactivation potential and pedestal current) than to interventions that change the rate of growth (time constants of activation and inactivation) or shape of the window \( I_{\text{Ca,L}} \) (slopes of inactivation and activation). Although EAD occurrence could be diminished with small changes in steady-state parameters (<5-mV shifts in half-activation and half-inactivation potential or reduction in the pedestal current; Fig. 7), kinetic parameters required order-of-magnitude changes to produce modest effects, often causing excessive changes in APD (Figs. 4–7 and S1).

Limitations and Implications

There are several limitations in this experimental–computational approach:

**Rabbit versus human ventricular myocytes.** This study was performed in rabbit ventricular myocytes, which differ in some respects from human myocytes. Although the results from this study need to be validated in human myocytes, previous studies have shown that the \( I_{\text{Ca,L}} \) properties are generally similar in both species (Grandi et al., 2010; Verkerk et al., 2011), suggesting that suppression of \( I_{\text{Ca,L}} \) noninactivating component could be an effective therapeutic strategy.

**Virtual \( I_{\text{Ca,L}} \).** To study how the biophysical properties of \( I_{\text{Ca,L}} \) affect EAD formation, it was required to replace the endogenous \( I_{\text{Ca,L}} \) with a computed, “virtual” \( I_{\text{Ca,L}} \), with tunable parameters under dynamic-clamp conditions. The current injected under the dynamic clamp, although incorporating \( \text{Ca}^{2+} \)-dependent inactivation in response to the virtual \( \text{Ca}^{2+} \) transient in the model, did not trigger SR \( \text{Ca}^{2+} \) release in the myocytes. Thus, endogenous \( \text{Ca}^{2+} \)-sensitive currents in the myocyte, such as \( \text{Na}^{+}/\text{Ca}^{2+} \) exchange and \( I_{Ks} \), were not activated. One consequence was that EADs reconstituted after virtual \( I_{\text{Ca,L}} \) injection were smaller than before nifedipine blockade (Fig. 2): this could be corrected by injecting additional \( I_{Ks} \) via the dynamic clamp to simulate \( \text{Ca}^{2+} \)-induced activation of \( I_{Ks} \) (Fig. 2E). Importantly, whether or not additional \( I_{Ks} \) were injected, reduction of the noninactivated pedestal was equally potent at suppressing EADs and restoring the APD.
Off-target effects of nifedipine. Our experimental approach required the full block of the endogenous I\textsubscript{Ca,L} to correctly evaluate the EAD sensitivity to the relatively small I\textsubscript{Ca,L} pedestal. We used 20 µM nifedipine, one of the most selective L-type Ca\textsuperscript{2+} channel blockers available. At the concentration used in this work, nifedipine may have had off-target effects, and possibly partially blocked I\textsubscript{K} conductance (Gotoh et al., 1991). This effect may have contributed to the reduction of EAD amplitude, as observed in some of the experiments under dynamic clamp (e.g., Fig. 2 D); however, we were able to effectively restore EAD amplitude by the addition of a fraction of the computed I\textsubscript{K} to the injected current (Fig. 2 E). We are confident that off-target effects of nifedipine have not biased the overall conclusion of this work, as the consequences of reducing I\textsubscript{Ca,L} pedestal current were also tested and confirmed in pure computer simulations, presented in Fig. S4.

Despite its limitations, we believe that this approach outlines a useful new strategy for drug discovery, potentially adaptable to high throughput screening of small molecules or genetic interventions, to identify new anti-arrhythmic agents. In this context, the dynamic-clamp approach represents a powerful method to move beyond simple screening for indiscriminate ion channel blockers and toward identifying and targeting subtle and selective aspects of ion channel biophysics to guide drug discovery.

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