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Authors
Madhvani, RV
Angelini, M
Xie, Y
et al.

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

Roshni V. Madhvani,1* Marina Angelini,1* Yuanfang Xie,7 Antonios Pantazis, Silvie Suriyani, Nils P. Borgstrom,2,4,5 Alan Garfinkel,2,4,5 Zhilin Qu,2,5 James N. Weiss,2,3,5 and Riccardo Olcese1,3,5,6

1Division of Molecular Medicine, Department of Anesthesiology, 2Department of Medicine (Cardiology), 3Department of Physiology, 4Department of Integrative Biology and Physiology, 5Cardiovascular Research Laboratory, and 6Brain Research Institute, David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, CA 90095
7Department of Pharmacology, University of California, Davis, Davis, CA 95616

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 (\(I_{\text{Ca,L}}\)) 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 \(I_{\text{Ca,L}}\) was replaced by a virtual \(I_{\text{Ca,L}}\) 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 \(I_{\text{Ca,L}}\) 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 (\(I_{\text{Ca,L}}\)) overlap, known as the \(I_{\text{Ca,L}}\) window current region (January et al., 1988). Within this membrane potential range, \(I_{\text{Ca,L}}\) 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 \(I_{\text{Ca,L}}\) 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 \(I_{\text{Ca,L}}\), suggesting that a reduction of the \(I_{\text{Ca,L}}\) window current may represent an effective maneuver to...
suppress EADs and normalize APD without blocking the early peak $I_{\text{Ca,L}}$ 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 $I_{\text{Ca,L}}$, with programmable properties into a cell in real time to study its effects on AP characteristics (Fig. 1E). 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) $I_{\text{Ca,L}}$ to identify whether additional parameters could be modified to suppress EAD formation and normalize APD. Accordingly, we systematically investigated the slopes of the voltage dependence of activation and inactivation, the nonactivating (or very slowly inactivating) late pedestal current, and the time constants of activation and inactivation, which shape the $I_{\text{Ca,L}}$ window current (Fig. 1, A and C) during the cardiac AP.

The results demonstrate that of all the $I_{\text{Ca,L}}$ 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 APD to reduce dispersion of repolarization: the half-activation and half-inactivation potentials and the nonactivating 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 $I_{\text{Ca,L}}$ 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 previously (Chen et al., 2003), and washed and bathed in Tyrode’s solution containing (mM): 136 NaCl, 5.4 KCl, 1 MgCl$_2$, 0.33 NaH$_2$PO$_4$, 1.8 CaCl$_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4. The intracellular solution contained (mM): 110 K-aspartate, 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 H$_2$O$_2$ 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 $I_{\text{Ca,L}}$ with the properties of the native $I_{\text{Ca,L}}$ is injected into the myocytes. To predict $I_{\text{Ca,L}}$ and its Ca$^{2+}$-dependent inactivation, our ventricular myocyte model also computes intracellular Ca$^{2+}$ cycling. To predict the spatiotemporal distribution of intracellular calcium, average [Ca$^{2+}$] was computed in four different cellular compartments, namely the “submembrane space” in proximity of the sarcolemma ($C_s$), 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 Ca$^{2+}$-regulated ionic conductance was also included in the model, i.e., the fast sodium current ($I_{\text{Na}}$), the Na$^+$/K$^+$ pump current ($I_{\text{pump}}$), the Na$^+$/Ca$^{2+}$ exchange current ($I_{\text{NCX}}$), and the Ca$^{2+}$-dependent slow component of the delayed rectifier potassium channel ($I_{\text{K}}$). Calcium-modulated currents sense the [Ca$^{2+}$] at submembrane space ($C_s$), which is higher than the global [Ca$^{2+}$] ($C_i$) (Weber et al., 2002). The average $C_i$ is used to calculate Ca$^{2+}$-dependent inactivation in the $I_{\text{Ca,L}}$ formulation, whereas $C_s$ was acquired during the course of the experiments to predict the amplitude and shape of the Ca$^{2+}$ transient.

In brief, the Ca$^{2+}$ flux into the cell caused by $I_{\text{Ca,L}}$ is given by:

$$I_{\text{Ca}} = g_{\text{Ca}} P_{\text{Ca}} i_{\text{Ca,L}}$$

(1)

where $C_i$ is the submembrane Ca$^{2+}$ concentration in units of millimolar, $P_{\text{Ca}}$ (0.00054 cm/s) is the Ca$^{2+}$ channel permeability, $V_m$ is the membrane potential, $F$ is the Faraday constant, and $T$ is temperature. $P_0$ was formulated as:

$$P_0 = d \cdot f \cdot q,$$

(3)

where $d$ is the voltage-dependent activation gate, $f$ is the VDI gate, and $q$ is the Ca$^{2+}$-dependent inactivation gate. The steady states of these gating variables as functions of the membrane potential ($V_m$) were formulated as follows:

$$d_a = \frac{1}{1.0 + \exp((V_m - d_{\text{half}})/d_{\text{slope}})}$$

(4)

$$f_a = \frac{1 - f_{\text{dest}}}{1.0 + \exp((V_m - f_{\text{half}})/f_{\text{slope}})} + f_{\text{dest}}$$

(5)

$$q_a = \frac{1}{1.0 + \left[\frac{C_i}{C_s}\right]^{\gamma}}$$

(6)

$$\tau_d = \frac{d_a \left(1 - \exp\left(-V_m - (d_{\text{half}} + a_d)\right)\right)}{a_d (d_{\text{slope}} + a_d) (V_m - (d_{\text{half}} + a_d))}$$

(7)

$$\tau_f = \frac{1}{b_f \exp\left(-b_f \left(V_m - (f_{\text{half}} + b_f)\right)^2\right) + b_f},$$

(8)
where $\text{dhalf}$ and $\text{fhalf}$ are the potentials at half-maximum of activation and inactivation, respectively; $p\text{dest}$ is the noninactivating pedestal of the inactivation gate; and $d\text{slope}/fs\text{lope}$ 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. $Cd$ is the submembrane [Ca$^{2+}$]; $Ca/M$ is the affinity for Ca$^{2+}$ of the inactivation gate; $z$ and $\gamma$ are the time constants of the $d$ gate and the $f$ gate, respectively; and $a_1$, $a_2$, $b_1$, $b_2$, $b_3$, and $b_4$ are additional factors used for fitting. The control parameters in the $\text{ICa,L}$ formulation were determined by fitting formulated current to experimental nifedipine-sensitive $\text{ICa,L}$ 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 $\text{ICa,L}$ 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 ($\text{IK}s$) was injected together with $\text{ICa,L}$. $\text{IK}s$ was modeled as in Mahajan et al. (2008).

Data analysis

APD at 90% repolarization (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 $\text{Ca}$ transient before and after reduction of $\text{ICa,L}$ pedestal (Fig. S2) under the dynamic clamp. Fig. S3 demonstrates that the reduction of $\text{ICa,L}$ 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 APD$_{90}$ for different $\text{ICa,L}$ activation and inactivation time constants. Table S2 shows the biophysical parameters of $\text{ICa,L}$ 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 $\text{ICa,L}$

In our previous study (Madhvani et al., 2011), we demonstrated that modest shifts (<5 mV) in the half-activation and half-inactivation potentials of $\text{ICa,L}$, 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 $\text{Ca}$ transient. However, other parameters also affect the $\text{ICa,L}$ 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 superfused with 600 µM H$_2$O$_2$ until EADs appeared consistently. The native $\text{ICa,L}$ was then blocked with 20 µM nifedipine, which eliminated EADs and markedly shortened the APD (Fig. 1 D). Next, the dynamic clamp was

addition of 20 µM nifedipine blocked the native $\text{ICa,L}$ (right). Under dynamic clamp (E), the myocyte membrane potential is fed into the model, which computes and injects a virtual $\text{ICa,L}$ into the cell in real time and reconstitutes EADs in the continuous presence of H$_2$O$_2$.

Figure 1. The $\text{ICa,L}$ window current region and the experimental design for dynamic clamp. (A) Changing steady-state biophysical properties of $\text{ICa,L}$, such as the half-activation potential ($V_{1/2}$ activation), the slope of the activation curve, or the noninactivating pedestal of the inactivation curve has a large impact on the $\text{ICa,L}$ window region, i.e., the area (shaded) subtended by the intersection of the voltage-dependent activation and inactivation curves. (B) The window region bounded by the product of the steady-state activation and steady-state inactivation curves (SS-act × SS-inact). (C) A typical rabbit ventricular myocyte AP after exposure to 600 µM H$_2$O$_2$, displaying a large-amplitude EAD. EADs often occur within the range of membrane potentials defined by the window current (around −50 to 20 mV). (D) Representative APs for the experimental protocol used in this study: cardiac myocytes (paced at 5 s; left) were exposed to 600 µM H$_2$O$_2$ to induce EADs (middle). The
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 µM 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 ~3 to ~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 C_a transient, which is important for activating I_K (Tohse, 1990; Nitta et al., 1994), we compensated by adding 10% of the computed I_K 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_K. Importantly, the reduction of the I_{Ca,L} pedestal suppresses the EAD regimen without attenuating the amplitude of the predicted C_a transient (Fig. S2).

These results indicate that the reduction of the non-inactivating 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](https://example.com/figure2.png)

**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 µM H_2O_2 to the bath solution–induced EADs within 5–10 min. (C) The addition of 20 µM 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_K. Note that both conditions regenerate the EAD regimen, although the amplitude of the oscillations tended to be larger with the addition of 10% I_K. (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 $I_{\text{Ca,L}}$ activation and inactivation

We next studied the effects of altering the time constants of $I_{\text{Ca,L}}$ activation and inactivation on EAD formation during oxidative stress. Overall, we found that changes in the rates of $I_{\text{Ca,L}}$ activation or inactivation by up to 10-fold had limited efficacy at suppressing EADs induced by H$_2$O$_2$ (Figs. 5 and 6).

Specifically, in the presence of H$_2$O$_2$, slowing $I_{\text{Ca,L}}$ activation by twofold ($\tau_a \times 2$) maintained an average APD$_{90}$ of $1,550 \pm 130$ ms, and EAD occurrence remained high at $91 \pm 4\%$ (Table S1). A 10-fold slowing of $I_{\text{Ca,L}}$ activation ($\tau_a \times 10$) prolonged APD$_{90}$ from $1,376 \pm 200$ ms to $2,711 \pm 548$ ms, whereas EAD occurrence increased from $76 \pm 8\%$ to $100\%$ of APs. Conversely, increasing the rate of $I_{\text{Ca,L}}$ activation by twofold ($\tau_a \times 0.5$; Table S1) or by 10-fold ($\tau_a \times 0.1$) had only a modest impact on both APD$_{90}$ ($1,122 \pm 126$ ms or $1,104 \pm 165$ ms) and EAD occurrence (79 ± 15\% or 77 ± 10\%), respectively (Fig. 5 and Table S1).

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 ($z = 25.6 e^0$) or $k = 8$ mV ($z = 3.2 e^0$), respectively. We did not observe a significant difference in either EAD amplitude or in percentage of APs with EADs (Fig. S1, C and D). APD$_{90}$ prolonged from $1,104 \pm 319$ ms to $3,140 \pm 771$ ms (Fig. S1, B and E) as the steepness of the inactivation curve was reduced from $k = 4$ mV ($z = 6.4 e^0$) to $k = 8$ mV ($z = 3.2 e^0$) (Fig. S1 A).

Sensitivity of EADs to the kinetics of $I_{\text{Ca,L}}$ activation

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When the rate of VDI in the dynamic-clamp model was increased by twofold ($\tau_d \times 0.5$), neither APD$_{90}$ (from the voltage dependence of activation (Fig. 4 A). We found that, at greater steepness, EAD amplitudes decreased from $5.6 \pm 0.7$ mV to $3.1 \pm 1$ mV (for $k = 2$ mV, $z = 12.8 e^0$) or $2.6 \pm 0.8$ mV ($k = 1$ mV, $z = 25.6 e^0$) (Fig. 4, B and C). Also, for the steepest slope ($k = 1$ mV, $z = 25.6 e^0$), 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 ($z = 6.4 e^0$) to $k = 6$ mV ($z = 4.3 e^0$), EAD amplitude increased from $5.6 \pm 0.7$ mV to $12.3 \pm 1.4$ mV (Fig. 4, B and C). This effect was even more pronounced for $k = 8$ mV ($z = 3.2 e^0$), such that the mean EAD amplitude increased to $21.4 \pm 2$ mV (Fig. 4, B and C), although no significant changes in percentage of APs with EADs were observed (Fig. 4 D), and APD$_{90}$ remained prolonged for these maneuvers (Fig. 4 E).

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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 types.
layer APs, respectively. When paced at a 5-s cycle length under control conditions, with a 3% \( I_{Ca,L} \) 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 \( H_2O_2 \) as described above, including increasing the \( I_{Ca,L} \) 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 \( I_{Ca,L} \) pedestal current was reduced to 0%, while maintaining all other \( H_2O_2 \) effects, APD\(_{90} \) shortened to 273, 293, and 309 ms, respectively, and EADs disappeared (Fig. S4 C). Thus, suppressing the \( I_{Ca,L} \) pedestal current was effective at eliminating EADs regardless of transmural AP heterogeneity.

**DISCUSSION**

Although many ionic currents can contribute to EAD formation, reactivation of \( I_{Ca,L} \) 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 \( H_2O_2 \) is likely to promote EADs by affecting multiple ionic currents such as late \( I_{Na} \) (Song et al., 2006), the modification of \( I_{Ca,L} \) is sufficient to prevent EADs even in the presence of \( H_2O_2 \). Furthermore, this intervention does not diminish the amplitude of the predicted \( Ca^{2+} \) transient (Fig. S2) and therefore is expected to maintain normal cell contractility. Conventional \( Ca^{2+} \) channel blockers such as nifedipine, which indiscriminately block both peak and window \( I_{Ca,L} \), are highly effective at suppressing EADs (e.g., Fig. 1 D). However, by blocking peak \( I_{Ca,L} \), 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_{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 Ca^{2+} channel agent for suppressing EAD-mediated arrhythmias (drugs or genetic intervention) would leave peak I_{Ca,L} hence excitation–contraction coupling, intact but selectively suppress late I_{Ca,L} in the window region. An important novel contribution of the present study is the finding that the I_{Ca,L} pedestal current has an equivalent promise to the half-activation and half-inactivation potentials as a novel anti-arrhythmic target to suppress EAD formation, which is robust across the spectrum of simulated transmural AP differences in the ventricular epicardial, M cell, and endocardial layers (Fig. S3). Moreover, it is effective for different mechanisms of EAD generation, as H_2 O_2 primarily causes EADs by increasing inward currents including late I_{Na} and I_{Ca,L} as a result of oxidative CaMKII activation (Ward and Giles, 1997; Xie et al., 2009; Wagner et al., 2011), whereas hypokalemia causes EADs by reducing K^- conductances, i.e., I_{Ks} and Na^+/K^+ ATPase activity, which in turn causes accumulation of intracellular Na^+ and Ca^{2+}. The finding that selective blockade of the I_{Ca,L} pedestal has potent EAD-suppressing effects is particularly intriguing because of the precedent in Na^- channels, in which selective blockers of the late Na^- current (I_{Na}), which leave the peak I_{Na} intact, are already in clinical use (Karwatowska-Prokopczuk et al., 2013). Given the overall structural similarities between Na^- and Ca^{2+} channels, the likelihood that analogous agents that selectively block late I_{Ca,L} can also be identified seems high. Moreover, because late I_{Na} can also play an important role in EAD formation (Xie et al., 2009; Yang et al., 2012), we can further speculate that the combination of a late I_{Na} blocker with a late I_{Ca,L} blocker might be a particularly efficacious anti-arrhythmic combination to prevent EAD-mediated arrhythmias.

The effects of modifying various I_{Ca,L} parameters on the APD and EAD occurrence observed in this study can be understood in terms of the dynamical theory of EAD formation as a dual Hopf-homoclinic bifurcation (Tran et al., 2009; Qu et al., 2013). In this theory, the membrane voltage oscillations characterizing EADs depend critically on the amount of time that the membrane potential dwells in the window region during repolarization. This dwell time must be long enough for the growth rate of I_{Ca,L} to overcome counterbalancing repolarizing currents such as I_{Ks}, thereby generating the upstroke of the EAD. As the membrane potential exceeds the voltage for peak I_{Ca,L} (≈0 mV), the diminishing amplitude of I_{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_{Ca,L} activation (Fig. 4), consistent with the dynamical theory stating that membrane oscillations (EADs) occur because of instability of the I_{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_{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_{Ca,L} (slopes of inactivation and inactivation). 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_{Ca,L} properties are generally similar in both species (Grandi et al., 2010; Verkerk et al., 2011), suggesting that suppression of I_{Ca,L} nonactivating component could be an effective therapeutic strategy.

Virtual I_{Ca,L}. To study how the biophysical properties of I_{Ca,L} affect EAD formation, it was required to replace the endogenous I_{Ca,L} with a computed, “virtual” I_{Ca,L} with tunable parameters under dynamic-clamp conditions. The current injected under the dynamic clamp, although incorporating Ca^{2+}-dependent inactivation in response to the virtual Ca^{2+} transient in the model, did not trigger SR Ca^{2+} release in the myocytes. Thus, endogenous Ca^{2+}-sensitive currents in the myocyte, such as Na^+/Ca^{2+} exchange and I_{Ks}, were not activated. One consequence was that EADs reconstituted after virtual I_{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 Ca^{2+}-induced activation of I_{Ks} (Fig. 2E). Importantly, whether or not additional I_{Ks} were injected, reduction of the nonactivated 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 \( I_{\text{Ca,L}} \) to correctly evaluate the EAD sensitivity to the relatively small \( I_{\text{Ca,L}} \) pedestal. We used 20 \( \mu M \) nifedipine, one of the most selective L-type \( Ca^{2+} \) channel blockers available. At the concentration used in this work, nifedipine may have had off-target effects, and possibly partially blocked \( I_{\text{Ca,L}} \) 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 \( I_{\text{K}} \) 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 \( I_{\text{Ca,L}} \) 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 antiarrhythmic 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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SUPPLEMENTAL METHODS

Computer simulations
The Markovian formulation for L-type Ca\(^{2+}\) channel open probability \(P_o\) was replaced by the Hodgkin–Huxley formulation, as used in the dynamic-clamp experiments described in the main text. The formulation of Ito was replaced by that of Dong et al. (2006) as modified by Zhao et al. (2012) to more accurately reflect experimental data on APD restitution. In the rabbit ventricular myocyte, the APD restitution curve is biphasic, as the APD at intermediate heart rates is greater than at fast and slow heart rates (Zhao et al., 2012). This has been attributed to the slow recovery of \(I_{to}\) in this type of cell (Bassani et al., 2004).

To model the effects of \(H_2O_2\), we made several changes to ionic currents (see below). A late \(I_{Na}\) current was implemented with a magnitude \(\frac{1}{1}\%\) of peak \(I_{Na}\) based on the findings of Song et al. (2010) of increased late \(I_{Na}\) in the presence of 200 µM \(H_2O_2\) in isolated rabbit ventricular myocytes.

The maximum conductance of \(I_{Ca,L}\) was increased by a factor of 2, which is within the range reported by Xie et al. (2009), who found that in rabbit ventricular myocytes, the peak \(I_{Ca,L}\) increased from 7.3 ± 0.8 pA/pF in a control environment to 12.1 ± 1.8 pA/pF in the presence of 1 mM \(H_2O_2\).

The maximum conductance of \(I_{to}\) was also increased by a factor of 1.57 based on experimental data from Zhao et al. (2012) on isolated rabbit ventricular myocytes. Finally, the maximum conductance of \(I_{NCX}\) was increased by a factor of 1.5 following the mathematical model of Zhao et al. (2012) (Table S3).

\(I_{Ca,L}\) formulation. The same model used for the dynamic clamp (see Materials and methods in the main text) was also used for computer simulations. Parameters for the control and \(H_2O_2\) models are shown in Table S2.

Late \(I_{Na}\) formulation. A late \(I_{Na}\) current was implemented by changing the formulation of \(I_{Na}\) to:

\[
I_{Na} = g_{Na}(m^hjR + \alpha)(V - E_{Na}),
\]

where \(\alpha\) reflects a noninactivating component of the channel. For the \(H_2O_2\) model, \(\alpha = 0.01\).

\(I_{to}\) formulation. The formulation of \(I_{to}\) was based on that of Dong et al. (2006), as modified by Zhao et al. (2012) to better fit rabbit ventricular myocytes:

\[
I_{to} = g_{to}(R + \alpha)(V - E_{to})
\]

\[
R = e^{V/500}
\]

\[
\alpha = \frac{4}{1 + e^{(V-250)/20}}, \beta = \frac{3.5}{1 + e^{(V-110)/20}}, m_x = \frac{1}{1 + e^{(V-110)/30}}
\]

\[
\alpha_h = \frac{0.016}{1 + e^{(V-50)/5.5}}, \beta_h = \frac{0.11}{1 + e^{(V+27)/6.5}}, h_x = \frac{1}{1 + e^{(V+50)/3.9}}
\]
**Figure S1.** Relevance of the slope of $I_{Ca,L}$ steady-state inactivation curve to EADs and APD. (A) Under dynamic clamp and in the presence of H$_2$O$_2$, we evaluated the effects of varying the slope ($k$) of the steady-state inactivation curve. The steady-state activation curve was kept constant, whereas the slope of the steady-state inactivation curve was varied under dynamic clamp from $k = 1$ mV to $k = 8$ mV. The pair of curves in the center, $k = 4$ mV, corresponds to the native $I_{Ca,L}$ modified by H$_2$O$_2$. (B) Representative APs obtained for each $k$ value studied. For each value of $k$ tested, the mean EAD amplitude (C), EAD occurrence (D), and APD$_{90}$ (E) are shown. Individual experiments are shown as closed circles, and the means for all experiments are plotted as open rectangles. Error bars indicate SEM. *, instances when the AP failed to repolarize before the next pacing stimulus are reported as RF (repolarization failure).

**Figure S2.** Predicted Ca transient before and after reduction of the $I_{Ca,L}$ pedestal. (A) A representative AP recorded in control conditions. (B) The predicted Ca transient upon AP clamp of the ventricular AP model with the AP waveform in A. (C) Representative AP recorded in dynamic clamp in the presence of H$_2$O$_2$ (10% $I_{Ca,L}$ pedestal), reconstituted EADs, and prolonged the APD. (D) The ventricular AP model predicts the Ca transient in real time during the AP recorded in C. (E) Upon a reduction in the $I_{Ca,L}$ pedestal to 4%, the AP is restored to $\sim$250 ms and EADs are abolished. (F) The ventricular AP model predicts the Ca transient in real time during the AP recorded in E. Note that the expected amplitude and shape of the Ca transient is also restored compared with control conditions shown in B.
Figure S3. Reducing the noninactivating $I_{Ca,L}$ pedestal effectively suppresses hypokalemia-induced EADs. Under dynamic clamp and hypokalemia (2.7 mM K$^+$), we evaluated the effect of lowering the noninactivating pedestal from 5 to ≤0.5% of the peak current. (A) Enlarged view of the steady-state activation and inactivation curves of $I_{Ca,L}$ shows the changes introduced to the noninactivating component (pedestal). (B) Representative AP recorded in dynamic clamp in the presence of hypokalemia at different values of pedestal. Lowering the pedestal value of $I_{Ca,L}$ from 5 to ≤0.5% caused EADs to be significantly reduced or completely abolished, and normal APD was restored (C and D). Individual experiments are shown as closed circles, and the means for all experiments are plotted as open rectangles. Error bars indicate SEM.

Figure S4. Reducing the noninactivating $I_{Ca,L}$ pedestal is an effective maneuver to suppress simulated H$_2$O$_2$-induced EADs in a computer model of rabbit ventricular cell layer types. (A) APs for M, endocardial, and epicardial versions of the Mahajan cell model under control conditions. (B) Modeling the effects of H$_2$O$_2$ produces AP prolongation and EADs in all cell models. (C) A reduction of the $I_{Ca,L}$ pedestal current from 6 to 0%, while maintaining other H$_2$O$_2$ effects, suppresses EADs and restores normal APDs in all cell models. See Materials and methods in the main text and supplemental text for detailed description of simulations.
**TABLE S1**

*EAD occurrence and APD<sub>90</sub> for changes in the time constant of I<sub>Ca,L</sub> activation and inactivation*

<table>
<thead>
<tr>
<th>Kinetic parameter modification</th>
<th>APs with EADs</th>
<th>APD&lt;sub&gt;90&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>τ&lt;sub&gt;i&lt;/sub&gt; × 0.1</td>
<td>77 ± 10%</td>
<td>1,104 ± 165</td>
</tr>
<tr>
<td>τ&lt;sub&gt;i&lt;/sub&gt; × 0.5</td>
<td>79 ± 15%</td>
<td>1,122 ± 126</td>
</tr>
<tr>
<td>τ&lt;sub&gt;i&lt;/sub&gt; × 1</td>
<td>76 ± 8%</td>
<td>1,376 ± 200</td>
</tr>
<tr>
<td>τ&lt;sub&gt;i&lt;/sub&gt; × 2</td>
<td>91 ± 4%</td>
<td>1,550 ± 130</td>
</tr>
<tr>
<td>τ&lt;sub&gt;i&lt;/sub&gt; × 10</td>
<td>100%</td>
<td>2,711 ± 548</td>
</tr>
<tr>
<td>τ&lt;sub&gt;j&lt;/sub&gt; × 0.1</td>
<td>85 ± 8%</td>
<td>2,433 ± 294</td>
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<tr>
<td>τ&lt;sub&gt;j&lt;/sub&gt; × 0.5</td>
<td>70 ± 16%</td>
<td>1,838 ± 435</td>
</tr>
<tr>
<td>τ&lt;sub&gt;j&lt;/sub&gt; × 1</td>
<td>76 ± 8%</td>
<td>1,376 ± 200</td>
</tr>
<tr>
<td>τ&lt;sub&gt;j&lt;/sub&gt; × 2</td>
<td>24 ± 14%</td>
<td>802 ± 183</td>
</tr>
<tr>
<td>τ&lt;sub&gt;j&lt;/sub&gt; × 10</td>
<td>73 ± 24%</td>
<td>1,506 ± 102</td>
</tr>
</tbody>
</table>

**TABLE S2**

*Biophysical parameters of I<sub>Ca,L</sub> in the dynamic-clamp model and computer model in control environment, H<sub>2</sub>O<sub>2</sub>, and hypokalemia conditions*

<table>
<thead>
<tr>
<th>I&lt;sub&gt;Ca,L&lt;/sub&gt; model fitting parameter</th>
<th>Control</th>
<th>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Hypokalemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>dhalf (mV)</td>
<td>−1.033</td>
<td>−5</td>
<td>−5</td>
</tr>
<tr>
<td>dslope (mV)</td>
<td>5.11</td>
<td>4.6</td>
<td>6.8</td>
</tr>
<tr>
<td>fbhalf (mV)</td>
<td>−18.65</td>
<td>−17</td>
<td>−17</td>
</tr>
<tr>
<td>fslope (mV)</td>
<td>5</td>
<td>4</td>
<td>3.9</td>
</tr>
<tr>
<td>pdest</td>
<td>0.03</td>
<td>0.1</td>
<td>0.05</td>
</tr>
<tr>
<td>Cα (mM)</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>Cst (mM)</td>
<td>0.11</td>
<td>0.02</td>
<td>0.11</td>
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<tr>
<td>a&lt;sub&gt;1&lt;/sub&gt;</td>
<td>10</td>
<td>6.36</td>
<td>10</td>
</tr>
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<td>4.24</td>
<td>0.097</td>
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<td>0.00692</td>
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<tr>
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<td>b&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.00968</td>
<td>0.012</td>
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### Table S3
Parameter values for maximal ionic conductances of $I_{Ca,L}$, $I_{NCX}$, and $I_{to}$ for control and $H_2O_2$ models of rabbit ventricular AP

<table>
<thead>
<tr>
<th>Ionic conductance parameter</th>
<th>Control</th>
<th>$H_2O_2$</th>
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</thead>
<tbody>
<tr>
<td>$G_{Ca,L}$</td>
<td>0.48</td>
<td>0.96</td>
</tr>
<tr>
<td>$G_{NCX}$</td>
<td>0.84</td>
<td>1.68</td>
</tr>
<tr>
<td>$G_{to}$</td>
<td>0.07</td>
<td>0.11</td>
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### Table S4
Original values and scaling factors of maximal conductance of $I_{to}$ and $I_{Ks}$ for Epi, Endo, and M cell models of rabbit ventricular AP

<table>
<thead>
<tr>
<th>Ionic conductance parameter</th>
<th>Original value</th>
<th>Scaling factor</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>$H_2O_2$</td>
</tr>
<tr>
<td>$G_{to}$</td>
<td>0.07</td>
<td>0.11</td>
</tr>
<tr>
<td>$G_{Ks}$</td>
<td>0.32</td>
<td>0.32</td>
</tr>
</tbody>
</table>
REFERENCES


