Repolarization Reserve Evolves Dynamically During the Cardiac Action Potential: Effects of Transient Outward Currents on Early Afterdepolarizations

Running title: Nguyen et al; $I_o$ and Early Afterdepolarizations

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Abstract:

**Background** - Transient outward K currents (I_{to}) have been reported both to suppress and facilitate early afterdepolarizations (EADs) when repolarization reserve is reduced. Here we used the dynamic clamp technique to analyze how I_{to} accounts for these paradoxical effects on EADs by influencing the dynamic evolution of repolarization reserve during the action potential.

**Methods and Results** - Isolated patch-clamped rabbit ventricular myocytes were exposed to either oxidative stress (H_{2}O_{2}) or hypokalemia to induce bradycardia-dependent EADs at a long pacing cycle length (PCL) of 6 s, when native rabbit I_{to} is substantial. EADs disappeared when the PCL was shortened to 1 s, when I_{to} becomes negligible due to incomplete recovery from inactivation. During 6-s PCL, EADs were blocked by the I_{to} blocker 4-aminopyridine, but reappeared when a virtual current with appropriate I_{to}-like properties was reintroduced using the dynamic clamp (n=141 trials). During 1-s PCL in the absence of 4-aminopyridine, adding a virtual I_{to}-like current (n=1,113 trials) caused EADs to reappear over a wide range of I_{to} conductance (0.005-0.15 nS/pF), particularly when inactivation kinetics were slow (t_{inact}≥20 ms) and the pedestal (non-inactivating component) was small (<25% of peak I_{to}). Faster inactivation or larger pedestals tended to suppress EADs.

**Conclusions** - Repolarization reserve evolves dynamically during the cardiac action potential. Whereas sufficiently large I_{to} can suppress EADs, a wide range of intermediate I_{to} properties can promote EADs by influencing the temporal evolution of other currents affecting late repolarization reserve. These findings raise caution in targeting I_{to} as an antiarrhythmic strategy.

**Key words:** arrhythmia, transient outward potassium current, repolarization reserve, early afterdepolarization, dynamic clamp
Introduction

Normal cardiac repolarization relies on a critical balance between depolarizing inward currents and repolarizing outward currents during the action potential (AP) plateau. Repolarization has built-in redundancy, or ‘reserve’, to protect against excessive AP duration (APD) lengthening and consequent QT interval prolongation. Repolarization reserve protects the heart against early afterdepolarizations (EADs) and triggered activities—both of which can promote ventricular arrhythmias such as torsade de pointes, polymorphic ventricular tachycardia and ventricular fibrillation. The concept of ‘reduced repolarization reserve’, originally formulated by Roden,\(^1\) summarizes conditions in which vulnerability to EAD-related arrhythmias increases due to a net decrease in repolarizing current—whether related to increased inward currents, decreased outward currents, or both. An intuitive commonly-held assumption is that all outward currents during the plateau phase increase repolarization reserve and thereby suppress EAD formation. However, recent experimental studies have shown that this is not always true for transient outward K currents (\(I_{to}\)). Although \(I_{to}\) suppressed EADs in atrial myocytes,\(^2\) \(I_{to}\) exacerbated EADs in ventricular myocytes with repolarization reserve reduced by oxidative stress.\(^3\) This seemingly paradoxical effect that an outward K current, which increases repolarization reserve, can exacerbate EADs has been explained theoretically\(^3,4\) as follows. By lowering the plateau voltage during the early phase 1 of the AP plateau, \(I_{to}\) delays the subsequent activation of other, slower time- and voltage-dependent outward currents (such as \(I_{Ks}\)), thus diminishing their contribution to repolarization reserve during phases 2 and 3 of the AP, and thereby facilitating EADs. The specific biophysical properties of \(I_{to}\) that determine whether it suppresses or promotes EADs, however, have not been systematically defined. This is an important concern given that drug therapy targeting \(I_{to}\) has been proposed as an antiarrhythmic and anti-heart failure...
therapy.

In this study, we used the dynamic clamp technique to experimentally define the arrhythmogenic ranges of three $I_{to}$ properties—maximum conductance, inactivation kinetics, and pedestal (here defined as the $I_{to}$ non-inactivating component). The dynamic clamp technique allows an $I_{to}$-like current with programmable properties to be introduced into a patch-clamped myocyte after the endogenous $I_{to}$ is blocked. In this fashion, we could systematically analyze how each specific biophysical characteristic of $I_{to}$-like currents promotes or suppresses EAD formation. This systematic analysis is an important advantage of the dynamic clamp because the properties of $I_{to}$ currents are quite diverse, both within and across species, with fast and slow voltage-dependent subtypes ($I_{to,1,r}$ and $I_{to,1,s}$) and a Ca-dependent current ($I_{to,2}$), all with differing kinetics. The dynamic clamp affords the opportunity to create virtual $I_{to}$ currents with properties covering this full spectrum, including human $I_{to}$ characteristics.

Our findings indicate that when overall repolarization reserve is reduced, $I_{to}$-like currents can promote EADs in rabbit ventricular myocytes over a wide range of conductances, particularly when the time constant of inactivation of $I_{to}$ is relatively slow (>20 ms) and its pedestal is small. Large $I_{to}$ conductances or large pedestals can also shorten APD and suppress EAD formation. These findings indicate that the repolarization reserve is not pre-determined at the onset of the action potential, but is a process that evolves dynamically during the entire AP plateau. This factor must be taken into account when designing antiarrhythmic strategies targeting $I_{to}$ or preventing EAD-mediated arrhythmias, particularly because we find that human $I_{to}$ properties fall within the range that can promote EADs.

**Methods**

An expanded methods section is available in the Supplemental Material.
Experimental animals and patch clamping

This study was approved by the UCLA Chancellor’s Animal Research Committee (ARC 2003-063-23C) and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with UCLA Policy 990 on the Use of Laboratory Animal Subjects in Research (revised 2010). From young adult (3- to 4-month-old) New Zealand white male rabbits (1.7-2.0 kg), single ventricular myocytes were freshly isolated for whole-cell patch clamp with unbuffered intracellular Ca and dynamic clamp studies as described previously. To induce EADs, H₂O₂ (0.2 or 1 mmol/L) was added to the superperfusate or the extracellular [K⁺] was reduced from 5.4 to 2.7 mmol/L. To inhibit Iₜ₀, 4-aminopyridine (4-AP; 2 mmol/L) was added to the perfusate.

Dynamic clamp technique and virtual Iₜ₀ formulation

Patch-clamped rabbit ventricular myocytes were injected with a programmable virtual Iₜ₀ using the dynamic clamp5,6 (10-kHz sampling frequency; real-time Linux-based software; www.rtxi.org). The virtual Iₜ₀, with instantaneous recovery from inactivation at -80 mV, was formulated as follows:

\[ I_{t_0} = G_{t_0} x_{t_0} (\alpha + (1 - \alpha) y_{t_0}) (V - E_t) \]  \hspace{1cm} \text{(Eq.1)}

\[ \tau_{y_{t_0}} = \tau_{\text{inact}} \left( \frac{1.0}{\left( 1.0 + e^{\frac{V+33.5}{10.0}} \right)} + 1.0 \right) \]  \hspace{1cm} \text{(Eq.2)}

Three parameters in the virtual Iₜ₀—the maximum conductance \( \bar{G}_{t_0} \), the inactivation time constant \( \tau_{\text{inact}} \), and the pedestal \( \alpha \)—were varied to simulate a wide range of features encompassing various Iₜ₀ subtypes. Pedestal is the non-inactivating Iₜ₀ component controlled by parameter \( \alpha \) (Eq.1).
Data and statistical analysis

Electrophysiological data were analyzed using Clampfit 10.4 (Axon instruments, Inc.) and OriginPro 9.0 SR2 (Microcal software, Inc.). In the statistical analysis of the contingency table, measures of association (odds ratio) and accuracy (sensitivity, specificity, positive and negative predictive values, positive and negative likelihood ratios) were obtained under a logistic regression model using generalized estimating equation (GEE) methods. GEE allows for non-independent (correlated) binary observations due to multiple observations from the same myocyte and rabbit (hierarchical structure). The 95% confidence intervals ([95% CI]) were computed using resampling (bootstrap) methods under this model. A $P$ value of $<0.05$ was considered significant.

Results

To investigate the biophysical properties that determine whether $I_{to}$ suppresses or promotes EADs, we formulated a virtual $I_{to}$ current to introduce into isolated patch-clamped rabbit ventricular myocytes using the dynamic clamp technique. The virtual $I_{to}$ has three independently-adjustable parameters (Supplemental Figure 1): maximal conductance $\bar{g}_{to}$, a single inactivation time constant $\tau_{\text{inact}}$, and a pedestal (a very slowly-inactivating or non-inactivating component defined here as % of peak $I_{to}$ that remains after 300 ms). Table 1 provides a literature review of experimentally-measured ranges of these $I_{to}$ parameters in normal and failing hearts from different species, including humans. These data include both the fast subtype $I_{to,1,f}$ encoded by Kv4.3/Kv4.2/KCHiP2 and the slow subtype $I_{to,1,s}$ encoded by Kv1.4. To cover the spectrum of the three parameter ranges in Table 1, as well as to approximate aggregate currents composed of multiple $I_{to}$ subtypes in the same cell or additional time-independent K currents that confer the
equivalent of a pedestal, we varied the virtual $I_{to}$ over a wide range, as follows: $G_{to}$ from 0.0005 to 5.0 nS/pF, $\tau_{inact}$ from 5 to 1200 ms, and the pedestal from 0 to 100% of peak $I_{to}$. The parameter ranges of the virtual $I_{to}$ also cover the reported features of both the human and rabbit $I_{to}$ currents.

**Blockade of endogenous $I_{to}$ suppresses EADs**

To induce EADs, patch-clamped rabbit ventricular myocytes were exposed to either oxidative stress with $H_2O_2$ (1 mmol/L) or ionic stress with moderate hypokalemia (2.7 mmol/L). With either stress, bradycardia-dependent EADs were consistently observed at a slow pacing cycle length (PCL) of 6 s (Figure 1B-C row 1).

EADs disappeared when $I_{to}$ was blocked, either by shortening the PCL to 1 s (Figure 1B-C row 2) or by application of the $I_{to}$ blocker 4-AP (2 mmol/L) during pacing at 6 s (Figure 1B-C row 3 & Figure 2). Suppression of EADs by $I_{to}$ blockade suggests that $I_{to}$ contributes to EAD formation under both stressed conditions. However, the evidence of EAD suppression by 4-AP is not definitive because 4-AP is not completely selective for $I_{to}$ such that 4-AP off-target effects could have also been responsible.

**$I_{to}$ reconstitution reverses EAD suppression by 4-AP**

To determine whether EAD suppression by 4-AP was related primarily to its blockade of $I_{to}$ rather than its off-target effects, we injected a virtual dynamic-clamp $I_{to}$ resembling the native rabbit $I_{to}$. In myocytes superfused with control Tyrode’s solution and paced at PCL 6 s or 1 s, EADs were never observed in the absence or presence of an injected virtual $I_{to}$ for any parameter combinations tested (46 trials, 6 myocytes, 5 rabbits). Thus, when repolarization reserve was normal, $I_{to}$ reconstitution did not promote EADs *de novo*.

However, after EADs had already been induced by either $H_2O_2$ or hypokalemia and subsequently suppressed by 4-AP, injection of a virtual $I_{to}$ with properties approximating the
native rabbit I_{to1-s} (Table 1) caused EADs to reappear (representative illustration in Figure 2, row 4). For the same value of $\bar{G}_{to}$, increasing the pedestal current from 0 to 50-55% of peak I_{to} caused EADs to disappear and APD to shorten markedly (Figure 2 row 5). Likewise, increasing $\bar{G}_{to}$ without increasing the pedestal had the same effect (Figure 2 row 6).

**I_{to} reconstitution reverses EAD suppression by rapid pacing**

To eliminate possible confounding off-target effects of 4-AP unequivocally, we took advantage of the fact that the native rabbit ventricular I_{to} (chiefly I_{to1-s}) has an unusually long time constant of recovery from inactivation, averaging 6 s at -80 mV. Thus, whereas I_{to} amplitude is substantial at a PCL of 6 s due to the long diastolic recovery interval between beats, I_{to} is almost completely inactivated and makes a negligible contribution to the AP at a PCL of 1 s.

Coincidentally, EADs induced by either oxidative stress or hypokalemia at PCL 6 s disappeared at PCL 1 s (Figure 1). These features allowed us to introduce a dynamic-clamp I_{to} programmed with instantaneous recovery from inactivation kinetics at -80 mV during PCL 1 s to determine if EADs reappear, and, if so, to evaluate what properties of I_{to} are required.

To assess the independent contribution of each of the three I_{to} parameters to EAD formation, we introduced a virtual I_{to}, varying only one parameter at a time (Figs 3-5), into myocytes in which stress-induced EADs at PCL 6 s had been suppressed by decreasing the PCL to 1 s.

Figure 3 illustrates the contribution of $\bar{G}_{to}$, assessed by altering $\bar{G}_{to}$ while holding $\tau_{inact}$ and the pedestal constant. In this representative myocyte, after hypokalemia-induced EADs were suppressed by pacing at 1 s (row 1), introducing a small virtual I_{to} ($\bar{G}_{to} = 0.01 \text{ nS/pF}, \tau_{inact} = 20 \text{ ms}, 0\% \text{ pedestal}$) caused APD to shorten (row 2). When $\bar{G}_{to}$ was increased to 0.05 nS/pF, EADs reappeared (row 3). Further increasing $\bar{G}_{to}$ to 0.15 nS/pF caused EADs to disappear and the APD
to shorten markedly (row 4). These findings demonstrate that for a given $\tau_{\text{inact}}$ and pedestal, $I_{\text{to}}$ promotes EADs over a critical range of $G_{\text{to}}$ and shortens APD at smaller or larger $G_{\text{to}}$ values beyond that critical range.

Figure 4 illustrates the contribution of $\tau_{\text{inact}}$, assessed by altering $\tau_{\text{inact}}$ while holding $G_{\text{to}}$ and the pedestal constant. In another myocyte, after $\text{H}_2\text{O}_2$-induced EADs were suppressed by pacing at 1 s (row 1), $I_{\text{to}}$ was reconstituted using the dynamic clamp ($G_{\text{to}} = 0.05 \text{ nS/pF, } \tau_{\text{inact}} = 20 \text{ ms, } 0\% \text{ pedestal}$). In this myocyte, $\tau_{\text{inact}}$ of 20 ms did not cause EADs to reappear, but shortened APD (row 2). Prolonging $\tau_{\text{inact}}$ to 80 or 100 ms, however, caused EADs to reappear (rows 3-4).

Figure 5 illustrates the contribution of the pedestal, assessed by altering the pedestal while holding $G_{\text{to}}$ and $\tau_{\text{inact}}$ constant. In this myocyte, after $\text{H}_2\text{O}_2$-induced EADs were suppressed by pacing at 1 s (row 1), introducing a virtual $I_{\text{to}}$ ($G_{\text{to}} = 0.025 \text{ nS/pF, } \tau_{\text{inact}} = 80 \text{ ms}$) with no pedestal caused EADs to re-emerge (row 2). EADs persisted (albeit with decreased frequency) when the pedestal was increased to 10, 25, and then 50% (rows 3-5). However, EADs disappeared when the pedestal was further increased to 75% (row 6).

$I_{\text{to}}$ properties that reverse EAD suppression by rapid pacing

Figure 6 summarizes results from 772 parameter combinations of $G_{\text{to}}, \tau_{\text{inact}},$ and pedestal values obtained in 1,113 trials using 131 rabbit ventricular myocytes isolated from a total of 46 rabbits. Using the protocols illustrated in Figures 3-5, we exposed myocytes to either $\text{H}_2\text{O}_2$ or hypokalemia at PCL 6 s to induce EADs, then suppressed EADs by shortening the PCL to 1 s, prior to injecting a virtual $I_{\text{to}}$. In the plots of $G_{\text{to}} \text{ vs. } \tau_{\text{inact}},$ parameter combinations of the virtual $I_{\text{to}}$ that caused EADs to reappear at PCL 1 s are indicated by solid symbols, while those that did not are indicated by open symbols. The four plots, labeled A-D, correspond to the size of the pedestal, which was 0% in A, 10-24% in B, 25-49% in C, and 50-75% in D.
Figure 6A shows that a wide range of \((\overline{G}_{t0}, \tau_{\text{inact}})\) parameter combinations (outlined by the dashed black line) caused EADs to reappear when the virtual \(I_{t0}\) had no pedestal component. The distribution of these parameter combinations agrees well with theoretical predictions from computer modeling simulating the effects of an \(I_{t0}\) with 0% pedestal on EAD formation.\(^3\) The gray-shaded area indicates the region in \((\overline{G}_{t0}, \tau_{\text{inact}})\) parameter space that caused EADs in the computer model. Note that the parameter combinations that caused EADs to reappear at PCL 1 s (the solid symbols) are mostly clustered inside the gray region, whereas those that did not (the open symbols) are mostly outside this gray area. This preferential clustering was statistically significant \((P<0.0001, \text{Table 2})\). The mismatches, indicated by the fraction of open symbols falling within the gray area or solid symbols outside the gray area, most likely reflect biological variability that does not exist in the deterministic computer AP model, as different patch-clamped myocytes came from different hearts and different ventricular regions in the same heart.

Figure 6B-D reveal the EAD-suppressing effects of pedestal current. As the pedestal component became larger, the region in the \((\overline{G}_{t0}, \tau_{\text{inact}})\) parameter space causing EADs to reappear (outlined by the colored regions) became progressively smaller, consistent with previously reported theoretical predictions.\(^3\) No EADs reemerged with pedestal current>75% (data not shown).

Finally, the red box in Figure 6B encloses parameter combinations representative of the human ventricular \(I_{t0}\) (predominantly \(I_{t0,1}\)) reported in the literature for both normal and failing human hearts (Table 1). Note that the human \(I_{t0}\) range falls within the virtual \(I_{t0}\) parameter region causing EADs to re-emerge.

In summary, the data in Figure 6 demonstrate that virtual \(I_{t0}\)-like currents can promote EADs over a wide range of peak conductances \((\overline{G}_{t0} \text{ over a 30-fold range from 0.005-0.15 nS/pF,})\)
especially when inactivation kinetics are slow ($\tau_{\text{inact}}>20$ ms) and the pedestal is small (<25% of peak $I_{to}$), in good overall agreement with theoretical predictions.\(^3\)

**Discussion**

**$I_{to}$ as friend and foe in EAD genesis**

Transient outward currents have been reported to both suppress and promote EADs.\(^2,3\) Because of the wide diversity of $I_{to}$ properties between different subtypes ($I_{to1,s}$, $I_{to1,f}$, and $I_{to2}$), marked differences in regional expression profiles within atrial and ventricular tissue in the same species, as well as marked interspecies differences (Table 1), the dynamic clamp technique offers a powerful tool to analyze how variations in $I_{to}$ properties affect EAD formation in diverse experimental settings. Moreover, the previous experimental evidence that $I_{to}$ promotes EADs has relied solely on the disappearance of EADs after applying 4-AP to block $I_{to}$. However, 4-AP is not completely selective for $I_{to}$ and has significant off-target effects on other ionic currents.\(^31,32\) The dynamic clamp allows the effects of $I_{to}$ on EADs to be tested directly without this complication.

The role of $I_{to}$ in EAD formation is a critical issue to understand because derangements in $I_{to}$ physiology have been linked to increased susceptibility to malignant arrhythmias and pharmacological strategies targeting this current are under development. In this context, both selective $I_{to}$ blockade and activation have been suggested as potential antiarrhythmic strategies.\(^2,18\) Downregulation of $I_{to1}$ contributes to reduced repolarization reserve in heart failure,\(^33\) which has been linked to higher risk of triggered ventricular arrhythmias.\(^34\) Genetic ablation of $I_{to1}$ in transgenic mice by $\text{Kv}1.4^{-}$ and $\text{Kv}4.2W362F$ crossbreeding\(^35\) or KChIP2 knockout\(^36\) significantly prolonged APD, promoting EADs in single ventricular myocytes\(^35\) and markedly prolonged QT interval, promoting spontaneous ventricular tachycardia in intact tissue.
in the absence of ventricular hypertrophy or heart failure. Potentiation of Ito to increase repolarization reserve has therefore been suggested as a potential antiarrhythmic strategy in heart failure, with the caveat that excessive Ito can cause arrhythmias by a different mechanism, namely phase 2 reentry as in Brugada syndrome and acute ischemia.

Our findings indicate that additional caution is warranted, since in addition to these potential pro-arrhythmic effects of excessive Ito, even mild to moderate augmentation of Ito may be proarrhythmic by promoting EADs when repolarization reserve is already compromised. However, we emphasize that when repolarization reserve was normal, adding a virtual Ito to the normal rabbit ventricular AP never induced EADs. Hence, for Ito to promote EADs, overall repolarization reserve must be reduced by additional factors, such as oxidative stress or hypokalemia. Also, we do not mean to imply that Ito is an absolute requirement for EADs. Rather Ito is a current whose properties can enable EADs that otherwise would not occur depending on specific (but common) electrophysiological conditions in multiple species.

Our systematic analysis of the Ito properties promoting EADs in this study also provides novel insights into the apparent discrepancy between Zhao et al’s study, which concluded that Ito promotes EADs in ventricular and Purkinje myocytes from multiple species, and Workman et al’s dynamic clamp study, which concluded that Ito suppresses EADs in rabbit and human atrial myocytes. Atrial tissue has a very large endogenous Ito that accounts for the triangular shape of its action potential. This large Ito is in the range that typically suppresses EADs (>0.10 to 0.15 nS/pF in Figure 6), such that reducing Ito by dynamic clamp subtraction brought Gto into the range that frequently promotes EADs (<0.10 nS/pF). Indeed, in Workman et al study, Gto averaged 0.34 nS/pF in rabbit atrial myocytes and 0.12 nS/pF in human atrial myocytes. In contrast, ventricular myocytes from most non-rodent mammals have a smaller Ito density (Table
1) that may place them in the range of $\tilde{g}_{Ito}$ that facilitates EAD formation such that $I_{to}$ block with 4-AP or other agents will suppress EADs. Rat and mouse ventricular myocytes, on the other hand, have higher $I_{to}$ densities than larger mammals, but may also develop EADs that are suppressed by 4-AP, suggesting that other differences between ventricular and atrial electrophysiology may also be important.

**Applicability to human $I_{to}$**

Our study is the first systematic analysis demonstrating that the range of $I_{to}$ properties capable of promoting EADs is wide and inclusive of $I_{to}$ from other species than just rabbit. We show that EADs were promoted over a 30-fold range of $I_{to}$ conductance, favored by an inactivation time constant $\tau_{inact} > 20$ ms and a pedestal component < 25% of peak $I_{to}$. This range includes the typical $I_{to}$ characteristics of both healthy and failing human ventricles, which exhibited a single inactivation time constant $\tau_{inact}$ averaging 8-75 ms and a pedestal of 16-22% in normal and failing epicardial ventricular myocytes. As shown in Figure 6B (red box), these characteristics fall clearly within the parameter range promoting EADs.

In addition, our findings also establish that the EAD-promoting effect of $I_{to}$ occurs not just when EADs are induced by oxidative stress with $H_2O_2$ (which significantly modified $I_{to}$ properties), but also when EADs are induced by the clinically relevant condition of moderate hypokalemia, a common complication of diuretic therapy in patients with heart failure. Hypokalemia induces EADs primarily by reducing outward K currents, whereas $H_2O_2$ augments the late Na current and Ca currents primarily through oxidative CaMKII activation (reflected in the more common appearance of DADs in association with $H_2O_2$-induced EADs, rather than with hypokalemia-induced EADs, as in Figure 2). Thus, the specific mechanism by which overall repolarization reserve is reduced does not appear to be critical to the ability of $I_{to}$ to
promote EADs. The overall implication is that $I_{to}$ may play an important role in facilitating EAD formation in multiple settings and in multiple species, including humans.

**Mechanism of EAD potentiation by $I_{to}$**

The mechanism by which $I_{to}$ potentiates EADs is consistent with the dynamic theory of EAD formation by a Hopf-homoclinic bifurcation mechanism. In this theory, EADs are generated by the opposing effects of inward $I_{CaL}$, which is activated as the plateau voltage dips below 0 mV and outward K currents, particularly $I_{Ks}$, which is reactivated during the $I_{CaL}$-mediated EAD upstroke. The activation-deactivation kinetics of $I_{Ks}$ must be matched appropriately to $I_{CaL}$ recovery kinetics to achieve membrane potential oscillations, the defining feature of EADs. If $I_{Ks}$ activates too rapidly, then repolarization rate is too fast for $I_{CaL}$ to reactivate and prevent repolarization. $I_{to}$ can promote EADs by lowering the voltage during the early plateau, thereby slowing $I_{Ks}$ activation (since $I_{Ks}$ activation rate and its open probability are highly voltage-dependent) while giving $I_{CaL}$ enough time to reactivate and oppose full repolarization. Thus, although $I_{to}$ always increases early repolarization reserve, the indirect effect of $I_{to}$ on the temporal evolution of other voltage-dependent currents such as $I_{Ks}$ and $I_{CaL}$ can paradoxically reduce late repolarization reserve, which is the critical phase during which EADs develop. This also explains why a larger $I_{to}$ pedestal current tends to suppress EADs, since the outward pedestal current directly increases late repolarization reserve, compensating for the reduction in $I_{Ks}$.

The agreement in Figure 6A between the computer model and the experimental findings lends further support to the dynamic theory of EAD formation via $I_{CaL}$ reactivation, even though other factors such as Ca cycling dynamics may also contribute importantly to EAD formation in many settings. The effects of Ca cycling might also account for the lack of an exact overlap between the computer model predictions (gray-shaded area) and the observed $I_{to}$ properties.
causing EADs to reappear in Figure 6A.

Finally, the findings in this study are also consistent with a previous study\(^5\) showing that fibroblast-myocyte coupling can promote EADs as a result of the \(I_{\text{to}}\)-like outward capacitive current introduced by the fibroblast into the myocyte through gap junctions during the early AP plateau phase. However, that myocyte-fibroblast gap junctional current also has a late sustained component that can become inward during the later phases of the action potential plateau, thus can further directly reduce late repolarization reserve.

**Study limitations**

To keep the number of parameters manageable, we simplified the virtual \(I_{\text{to}}\) formulation to include only a single inactivation time constant \(\tau_{\text{inact}}\), whereas two time constants have been reported in some studies, although not in humans (Table 1). A pedestal component was used to approximate long time constants of inactivation (>200 ms) as well as truly non-inactivating components. In addition, under some conditions, time-independent K currents, such as the plateau K current (\(I_{\text{Kp}}\)) or the ATP-sensitive K current (\(I_{\text{KATP}}\)), can potentially contribute a sustained outward current during the plateau phase that may summate with the \(I_{\text{to}}\) pedestal current.

We did not explicitly test scenarios in which multiple virtual \(I_{\text{to}}\) currents with different parameter combinations were added together into the same myocyte, even though multiple \(I_{\text{to}}\) subtypes (\(I_{\text{to1f}}, I_{\text{to1s}}, \text{and } I_{\text{to2}}\)) can coexist in the same myocyte. However, the wide range of parameter combinations that we tested, which exceeded the experimentally measured range in Table 1, would approximate many of these potential cases. Unlike \(I_{\text{to1}}, I_{\text{to2}}\) is not a K current, but a Ca-activated Cl current with time course that parallels the intracellular Ca transient.\(^{45,46}\)

Nevertheless, since \(I_{\text{to2}}\) activates rapidly and inactivates within 20-50 ms,\(^{45,46}\) its kinetics as an...
outward current fall within the range of virtual $I_{to}$ parameter combinations that we tested. Similarly, recent evidence indicates that small Ca-activated K (SK) channels are present in normal atrium and failing ventricles. $^{47,48}$ These SK currents track the Ca transient, similar to $I_{to2}$, and likewise are expected to fall within the parameter ranges that we tested. Given these limitations, however, our virtual $I_{to}$ model with three independently adjustable parameters should be viewed as a rough guideline for identifying the key EAD-promoting characteristics of $I_{to}$-like currents.

Although the ranges of virtual $I_{to}$ parameters in this study encompasses the endogenous $I_{to}$ parameter ranges from multiple species, the caveat is that we injected the virtual $I_{to}$ only into rabbit ventricular myocytes. Therefore, we cannot exclude the possibility that myocytes from other species, including humans, or myocytes remodeled by heart diseases, would behave differently. However, we believe that the differences would likely be quantitative rather than qualitative given that Zhao et al $^3$ found that $I_{to}$ block with 4-AP suppressed $H_2O_2$-induced EADs in multiple species exhibiting markedly different action potential properties.

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**Conflict of Interest Disclosures:** None
References:


Table 1: Literature review of ventricular I\textsubscript{to1} parameters measured from different species. I\textsubscript{to1} density was reported either as peak or as difference between peak and pedestal. Inactivation kinetics were reported as a mono- (\(\tau_1\)) or bi-exponential (\(\tau_1, \tau_2\)) decay time course.

<table>
<thead>
<tr>
<th>Species</th>
<th>I\textsubscript{to1} Subtype</th>
<th>Temp (°C)</th>
<th>Density (pA/pF)</th>
<th>G\textsubscript{i0} (nS/pF)</th>
<th>(\tau_{\text{inact}}) (ms)</th>
<th>Pedestal (% of peak)</th>
<th>References</th>
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<td>Human</td>
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<td></td>
<td>I\textsubscript{to1},f</td>
<td>21-24</td>
<td>8-14</td>
<td>0.06-0.11</td>
<td>46-75</td>
<td>16-22</td>
<td>7-10</td>
</tr>
<tr>
<td></td>
<td>I\textsubscript{to1},f</td>
<td>21-24</td>
<td>↓ ↔</td>
<td>↓ ↔</td>
<td>-</td>
<td>-</td>
<td>8-12</td>
</tr>
<tr>
<td></td>
<td>I\textsubscript{to1},s</td>
<td>21-24</td>
<td>5-10</td>
<td>0.04-0.08</td>
<td>59-73</td>
<td>0-17</td>
<td>8-12</td>
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<tr>
<td>Rabbit</td>
<td>I\textsubscript{to1},s</td>
<td>34-37</td>
<td>10-18</td>
<td>0.08-0.14</td>
<td>10-20</td>
<td>4-25</td>
<td>3,13-17</td>
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<tr>
<td></td>
<td>I\textsubscript{to1},s</td>
<td>25</td>
<td>33-38</td>
<td>0.25-0.29</td>
<td>30-35</td>
<td>15</td>
<td>17</td>
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<tr>
<td></td>
<td>I\textsubscript{to1},s</td>
<td>34-37</td>
<td>6-27</td>
<td>0.05-0.21</td>
<td>(\tau_1: 7-8, \tau_2: 70-118)</td>
<td>5-21</td>
<td>13-17,17</td>
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<tr>
<td></td>
<td>I\textsubscript{to1},s</td>
<td>25</td>
<td>8</td>
<td>0.06</td>
<td>38-50</td>
<td>24</td>
<td>17</td>
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<tr>
<td>H\textsubscript{2}O\textsubscript{2}</td>
<td>I\textsubscript{to1},s</td>
<td>37</td>
<td>13</td>
<td>0.10</td>
<td>(\tau_1: 65-80, \tau_2: 350)</td>
<td>35</td>
<td>3</td>
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<tr>
<td>Canine</td>
<td>I\textsubscript{to1}</td>
<td>36-37</td>
<td>17-46</td>
<td>0.13-0.35</td>
<td>18-36</td>
<td>-</td>
<td>3,9,18-23</td>
</tr>
<tr>
<td></td>
<td>I\textsubscript{to1}</td>
<td>24</td>
<td>20-22</td>
<td>0.15-0.17</td>
<td>34-49</td>
<td>0-16</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>I\textsubscript{to1}</td>
<td>36-37</td>
<td>5-13</td>
<td>0.04-0.10</td>
<td>22-43</td>
<td>0-69</td>
<td>18-20,23</td>
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<tr>
<td>Rat</td>
<td>I\textsubscript{to1},f</td>
<td>37</td>
<td>20-22</td>
<td>0.15-0.17</td>
<td>45-97</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>I\textsubscript{to1},f</td>
<td>20-25</td>
<td>9-39</td>
<td>0.07-0.30</td>
<td>16-55</td>
<td>7-40</td>
<td>21,25-28</td>
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<td>LVH</td>
<td>I\textsubscript{to1},f</td>
<td>20-25</td>
<td>2-40</td>
<td>0.02-0.31</td>
<td>35-81</td>
<td>11-36</td>
<td>25-27,29,30</td>
</tr>
<tr>
<td>Chronic MI</td>
<td>I\textsubscript{to1},f</td>
<td>37</td>
<td>11-12</td>
<td>0.08-0.09</td>
<td>45-97</td>
<td>-</td>
<td>24</td>
</tr>
</tbody>
</table>

Temp: recording temperature; LVH: left ventricular hypertrophy; MI: myocardial infarction
Table 2: Statistical analysis of predicted vs. observed (G_{to}, \tau_{\text{inact}}) parameter combinations causing EADs to reappear. A total of 480 observed (G_{to}, \tau_{\text{inact}}) combinations of a virtual I_{to} with no pedestal (787 trials in 79 ventricular myocytes from 25 rabbit hearts) that did (EAD^+) or did not (EAD^-) cause H_2O_2-induced or hypokalemia-induced EADs to reappear at PCL 1 s are compared to theoretical predictions from a computer model.

<table>
<thead>
<tr>
<th>No. of G_{to}-\tau_{\text{inact}} Combinations</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EAD^+</td>
</tr>
<tr>
<td>Predicted</td>
<td>186</td>
</tr>
<tr>
<td></td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>208</td>
</tr>
</tbody>
</table>

Positive Predictive Value [95% CI] = 0.77 [0.67, 0.87]
Negative Predictive Value [95% CI] = 0.93 [0.86, 0.99]
Sensitivity [95% CI] = 0.92 [0.82, 0.99]
Specificity [95% CI] = 0.72 [0.55, 0.89]
Positive Likelihood Ratio = 3.3 [1.21, 5.41]
Negative Likelihood Ratio = 0.11 [0.01, 0.26]
Odds Ratio [95% CI] = 41.5 [1.8, 84.9]

P < 0.0001
Figure Legends:

**Figure 1:** $I_{to}$ blockade by rapid pacing at PCL 1 s or by 4-AP suppresses $H_2O_2$-induced and hypokalemia-induced EADs in rabbit ventricular myocytes. A. No EADs arose under control conditions at PCL 6 s, 1 s, or 6 s in the presence of 4-AP (2 mmol/L). B-C. Following exposure to $H_2O_2$ (1 mmol/L; B) or hypokalemia (2.7 mmol/L; C), EADs (*) occurred at PCL 6 s (row 1), but were suppressed by rapid pacing at PCL 1 s (row 2) or by adding 4-AP (row 3). Superimposed action potentials under the 3 conditions are shown below.

**Figure 2:** Virtual $I_{to}$ reconstitutes $H_2O_2$-induced (A) and hypokalemia-induced (B) EADs blocked by 4-AP. Row 1: Action potentials were elicited during pacing at PCL 6 s under control conditions. Row 2: $H_2O_2$ (1 mmol/L) or hypokalemia (2.7 mmol/L) induced EADs (*) during slow pacing at 6 s. Row 3: $I_{to}$ blockade with 4-AP (2 mmol/L) suppressed EADs. Row 4: Representative parameter combinations of the virtual $I_{to}$ that caused EADs to reappear. Rows 5-6: Representative parameter combinations of the virtual $I_{to}$ that shortened APD and suppressed EAD reappearance by increasing the pedestal or $I_{to}$ conductance.

**Figure 3:** Effects of $\tilde{g}_{to}$ on reappearance of pacing-suppressed EADs. Row 1: EADs induced by hypokalemia (2.7 mmol/L) at PCL 6 s (not shown) were suppressed by shortening PCL to 1 s. Rows 2-4: A virtual $I_{to}$ with $\tau_{inact} = 25$ ms and no pedestal reconstituted EADs (*) at an intermediate $I_{to}$ conductance ($\tilde{g}_{to} = 0.05$ nS/pF, row 3), whereas smaller (0.01 nS/pF, row 2) or larger conductances (0.15 nS/pF, row 4) caused APD shortening.
**Figure 4:** Effects of \( \tau_{\text{inact}} \) on reappearance of pacing-suppressed EADs. Row 1: EADs induced by H\(_2\)O\(_2\) (1 mmol/L) at PCL 6 s (not shown) were suppressed by shortening PCL to 1 s. Rows 2-4: A virtual I\(_{\text{to}}\) with \( \overline{G}_{\text{to}} = 0.05 \) nS/pF and no pedestal did not reconstitute EADs (*) for \( \tau_{\text{inact}} = 20 \) ms (row 2), but did when \( \tau_{\text{inact}} \) was prolonged to 80 (row 3) or 100 ms (row 4).

**Figure 5:** Effects of the pedestal component on the reappearance of pacing-suppressed EADs. Row 1: EADs induced by H\(_2\)O\(_2\) (1 mmol/L) at PCL 6 s (not shown) were suppressed by shortening PCL to 1 s. Rows 2-6: A virtual I\(_{\text{to}}\) with \( \overline{G}_{\text{to}} = 0.025 \) nS/pF and \( \tau_{\text{inact}} = 80 \) ms reconstituted EADs (∗) for pedestals up to 50% (rows 2-5), but not for a pedestal of 75% (row 6).

**Figure 6:** Virtual I\(_{\text{to}}\) parameter combinations causing pacing-suppressed H\(_2\)O\(_2\)-induced or hypokalemia-induced EADs to reappear. Graphs show \( (\overline{G}_{\text{to}}, \tau_{\text{inact}}) \) combinations that did (solid circles) or did not (open circles) cause EADs to reappear at PCL 1 s, using the protocols shown in Figures 3-5, for the different ranges of I\(_{\text{to}}\) pedestal components as indicated in A-D. A. Dashed black line outlines the border of the experimental region in parameter space causing EADs to reappear, compared to the predictions from a computer model (gray shaded area), adapted from Zhao et al.\(^3\) B-D. Solid colored regions outline the experimental region in parameter space causing EADs to reappear for pedestals ranging from 10-24% (B), 25-49% (C), and 50-75% (D), compared to the no-pedestal case (black line reproduced from A). In B, the red box indicates the typical parameter values for human ventricular I\(_{\text{to},f}\) in normal and failing hearts (see Table 1). No EADs re-emerged with pedestals>75%.
$H_2O_2$

$H_2O_2 + \text{virtual } I_{to}$

- $\tau_{inact} = 20 \text{ ms}$
- $\tau_{inact} = 80 \text{ ms}$
- $\tau_{inact} = 100 \text{ ms}$
Repolarization Reserve Evolves Dynamically During the Cardiac Action Potential: Effects of Transient Outward Currents on Early Afterdepolarizations
Thao P. Nguyen, Neha Singh, Yuanfang Xie, Zhilin Qu and James N. Weiss

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SUPPLEMENTAL METHODS

Experimental animals
Young adult (3- to 4-month-old) New Zealand white male rabbits (1.7-2.0 kg) were injected intravenously once with heparin sulfate (1,000 U) and sodium pentobarbital (100 mg/kg). Following confirmation of adequate anesthesia (absence of pedal withdrawal reflex, corneal reflex, and motor response to pain stimuli by scalpel tip), hearts were rapidly excised and perfused for myocyte isolation.

Patch clamp methods
Freshly isolated single ventricular myocytes were used within 8 h for whole-cell patch clamp studies and dynamic clamp as described previously. The EGTA-free standard pipette solution contained (in mmol/L) K-aspartate 110, KCl 30, NaCl 5, HEPES 10, MgATP 5, creatine phosphate 5, and cAMP 0.1 (pH 7.2 adjusted with KOH). Cells were superfused at 37°C with standard Tyrode's solution containing (in mmol/L) NaCl 136, KCl 5.4 (or 2.7 in hypokalemia experiments), NaH₂PO₄ 0.33, CaCl₂ 1.8, MgCl₂ 1, HEPES 10, and glucose 10 (pH 7.4 adjusted with NaOH) unless otherwise indicated. Corrections were made for liquid junction potential (-13 mV). Action potentials were elicited in the current clamp mode at a pacing cycle length (PCL) of 1 or 6 s by 2-ms current pulses of at least twice threshold. Data were acquired and filtered at 2 kHz (Axopatch 200B patch-clamp amplifier; Digidata 1200 acquisition board; and Clampex 8.0, Axon Instruments, Inc.) then analyzed using Clampfit 9.2 (Axon instruments, Inc.) and Origin 7.5 (Microcal Software, Inc.). To induce EADs, H₂O₂ (0.2 or 1 mmol/L) was added to the superfusate or the extracellular [K] was reduced from 5.4 to 2.7 mmol/L. To inhibit Iₒ, 4-aminopyridine (4-AP; 2 mmol/L) was added to the perfusate.
Dynamic clamp technique and virtual $I_{to}$ formulation

The virtual $I_{to}$ used in our dynamic clamp experiment was modified from the $I_{to}$ formulation described in the rabbit ventricular model by Mahajan et al,\(^2\) as follows:

\[
I_{to} = G_{to} x_{tof} (\alpha + (1 - \alpha) y_{tof})(V - E) \tag{Eq.1}
\]

\[
\tau_{ytot} = \tau_{inact} \left( \frac{1.0}{(1.0 + e^{43.5})} + 1.0 \right) \tag{Eq.2}
\]

Specifically, we introduced a pedestal current controlled by parameter $\alpha$ in Eq.1 to simulate a very slowly- or non-inactivating component. We also controlled the inactivation time constant $\tau_{ytot}$ by varying $\tau_{inact}$ (Eq.2).

Computational modeling

To generate EADs in our computer simulations, we simulated the $\text{H}_2\text{O}_2$-induced EADs by replacing the Markovian L-type Ca current ($I_{CaL}$) in the Mahajan et al\(^2\) rabbit ventricular myocyte model with a Hodgkin-Huxley formulation based on the 1994 Luo and Rudy model\(^3\) fitted to the experimentally-measured properties of $I_{Ca,L}$ after $\text{H}_2\text{O}_2$ exposure.\(^4\) The maximal $I_{CaL}$ flux was 306 mmol-cm\(^{-1}\)-C\(^{-1}\) and the peak $I_{NCX}$ conductance was 1.26 nS/pF. Since $\text{H}_2\text{O}_2$ is known to activate late $I_{Na}$,\(^5\) a 2.1% late Na current was added by modifying the inactivation gates of the Na channel the same way as in Eq.1.
Supplemental Figure 1. Virtual $I_{to}$ trajectories. (A) Virtual $I_{to}$ currents (red traces) with $\tau_{inact} = 250 \text{ ms}$, pedestal of 25%, and variable $\bar{G}_{to}$ of 0.04, 0.05, and 0.06 nS/pF reconstituted EADs (*) in this representative rabbit ventricular myocyte. (B). Comparison of the three virtual $I_{to}$ currents with different $\bar{G}_{to}$ values from experiments illustrated in (A).
SUPPLEMENTAL REFERENCES


