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Repolarization Reserve Evolves Dynamically During the Cardiac Action Potential

Effects of Transient Outward Currents on Early Afterdepolarizations

Thao P. Nguyen, MD, PhD; Neha Singh, PhD; Yuanfang Xie, PhD; Zhilin Qu, PhD; James N. Weiss, MD

**Background**—Transient outward K currents (I\textsubscript{to}) have been reported both to suppress and to facilitate early afterdepolarizations (EADs) when repolarization reserve is reduced. Here, we used the dynamic clamp technique to analyze how I\textsubscript{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\textsubscript{2}O\textsubscript{2}) or hypokalemia to induce bradycardia-dependent EADs at a long pacing cycle length of 6 s, when native rabbit I\textsubscript{to} is substantial. EADs disappeared when the pacing cycle length was shortened to 1 s, when I\textsubscript{to} becomes negligible because of incomplete recovery from inactivation. During 6-s pacing cycle length, EADs were blocked by the I\textsubscript{to} blocker 4-aminopyridine, but reappeared when a virtual current with appropriate I\textsubscript{to}-like properties was reintroduced using the dynamic clamp (n=141 trials). During 1-s pacing cycle length in the absence of 4-aminopyridine, adding a virtual I\textsubscript{to}-like current (n=1113 trials) caused EADs to reappear over a wide range of I\textsubscript{to} conductance (0.005–0.15 nS/pF), particularly when inactivation kinetics were slow (τ\textsubscript{inac}≥20 ms) and the pedestal (noninactivating component) was small (<25% of peak I\textsubscript{to}). Faster inactivation or larger pedestals tended to suppress EADs.

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

**Key Words:** arrhythmias, cardiac ■ dynamic clamp ■ early afterdepolarization ■ repolarization reserve ■ transient outward potassium current

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) shortening and consequent QT interval prolongation. Repolarization reserve protects the heart against early afterdepolarizations (EADs) and triggered activity: 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,\textsuperscript{1} summarizes conditions in which vulnerability to EAD-related arrhythmias increases because of 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\textsubscript{to}). Although I\textsubscript{to} suppressed EADs in atrial myocytes,\textsuperscript{2} I\textsubscript{to} exacerbated EADs in ventricular myocytes with repolarization reserve reduced by oxidative stress.\textsuperscript{3} This seemingly paradoxical effect that an outward K current, which increasing repolarization reserve, can exacerbate EADs has been explained theoretically\textsuperscript{1,4} as follows. By lowering the plateau voltage during the early phase 1 of the AP plateau, I\textsubscript{to} delays the subsequent activation of other, slower time- and voltage-dependent outward currents (such as I\textsubscript{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\textsubscript{to} that determine whether it suppresses or promotes EADs, however, have not been systematically defined. This is an important concern.

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WHAT IS KNOWN

- Outward currents generally increase repolarization reserve and suppress early afterdepolarizations.
- Downregulation of the transient outward K current (I_{to}) contributes to the reduction in repolarization reserve during heart failure, thereby predisposing failing hearts to early afterdepolarizations—mediated arrhythmias.

WHAT THE STUDY ADDS

- Using the dynamic clamp technique to introduce a virtual I_{to} into rabbit ventricular myocytes, we show that because I_{to} inactivates before the end of the action potential, it can induce, rather than suppress, early afterdepolarizations, over a wide variety of conductances and kinetic properties.
- These findings demonstrate that repolarization reserve is a dynamic property that evolves during the action potential plateau, such that increases in early repolarization reserve can paradoxically decrease late repolarization reserve and promote early afterdepolarizations.
- I_{to} restoration in heart failure should be viewed with caution as an antiarrhythmic strategy.

Designing antiarrhythmic strategies 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 3 I_{to} properties: maximum conductance, inactivation kinetics, and pedestal (here defined as the I_{to} noninactivating 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 diverse, both within and across species, with fast and slow voltage-dependent subtypes (I_{to,f} and I_{to,s}) and a Ca-dependent current (I_{to,c}), 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 repolarization reserve is not predetermined at the onset of the AP, 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 Data Supplement.

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 (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, H_{2}O_{2} (1 mmol/L) was added to the superfusate or the extracellular [K] was reduced from 5.4 to 2.7 mmol/L. To inhibit I_{to}, 4-aminopyridine (4-AP; 2 mmol/L) was added to the perfusate.

Dynamic Clamp Technique and Virtual I_{to} Formulation

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

\[ I_{to} = \bar{G}_{to} \cdot x_{to} \left( \alpha + (1 - \alpha) \cdot y_{to} \right) \left( V - E_{k} \right) \]

\[ \tau_{inact} = \frac{1.0}{\left( 1.0 + e^{\frac{V + 33.5}{100.0}} \right) + 1.0} \]

Three parameters in the virtual I_{to} namely the maximum conductance \( \bar{G}_{to} \), the inactivation time constant \( \tau_{inact} \), and the pedestal \( \alpha \), were varied to simulate a wide range of features encompassing various I_{to} subtypes. Pedestal is the noninactivating I_{to} component controlled by parameter \( \alpha \) (Equation 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 methods. Generalized estimating equation allows for nonindependent (correlated) binary observations because of multiple observations from the same myocyte and rabbit (hierarchical structure). The 95% confidence intervals 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 3 independently adjustable parameters (Figure 1 in the Data Supplement): maximal conductance \( \bar{G}_{to} \), a single inactivation time constant \( \tau_{inact} \), and a pedestal (a slowly
inactivating or noninactivating 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 \( I_{to,f} \) encoded by Kv4.3/Kv4.2/KC3H2 and the slow subtype \( I_{to,s} \) encoded by Kv1.4. To cover the spectrum of the 3 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 \( \text{H}_2\text{O}_2 \) (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 and 1C, row 1).

EADS disappeared when \( I_{to} \) was blocked, either by shortening the PCL to 1 s (Figure 1B and 1C, row 2) or by application of the \( I_{to} \) blocker 4-AP (2 mmol/L) during pacing at 6 s (Figure 1B and 1C, row 3 and 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

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**Table 1. Literature Review of Ventricular \( I_{to} \) Parameters Measured From Different Species**

<table>
<thead>
<tr>
<th>Species</th>
<th>( I_{to} ) Subtype</th>
<th>Temp (°C)</th>
<th>Density (pA/pF)</th>
<th>( G_{to} ) (nS/pF)</th>
<th>( \tau_{inact} ) (ms)</th>
<th>Pedestal (% of Peak)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Normal ( I_{to,f} )</td>
<td>35</td>
<td>…</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>7–10</td>
</tr>
<tr>
<td></td>
<td>Normal ( I_{to,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>Heart failure ( I_{to,f} )</td>
<td>21–24</td>
<td>↓ ↔</td>
<td>↓ ↔</td>
<td>…</td>
<td>8–12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heart failure ( I_{to,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>
</tr>
<tr>
<td>Rabbit</td>
<td>Normal ( I_{to,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>
</tr>
<tr>
<td></td>
<td>Normal ( I_{to,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>
</tr>
<tr>
<td></td>
<td>Heart failure ( I_{to,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</td>
</tr>
<tr>
<td></td>
<td>Heart failure ( I_{to,s} )</td>
<td>25</td>
<td>8</td>
<td>0.06</td>
<td>38–50</td>
<td>24</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>( \text{H}_2\text{O}_2 )</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>
</tr>
<tr>
<td>Canine</td>
<td>Normal ( I_{to} )</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>Normal ( I_{to} )</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>Heart failure ( I_{to} )</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>
</tr>
<tr>
<td>Rat</td>
<td>Normal ( I_{to,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>Normal ( I_{to,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>
</tr>
<tr>
<td></td>
<td>LVH ( I_{to,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></td>
<td>Chronic MI ( I_{to,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>

\( I_{to} \) density was reported either as peak or as difference between peak and pedestal. Inactivation kinetics were reported as a mono- (\( \tau_1 \)) or biexponential (\( \tau_1 \), \( \tau_2 \)) decay time course. LVH indicates left ventricular hypertrophy; MI, myocardial infarction; and Temp, recording temperature.

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**Figure 1.** \( I_{to} \) blockade by rapid pacing at pacing cycle length (PCL) 1 s or by 4-aminopyridine (4-AP) suppresses \( \text{H}_2\text{O}_2 \)-induced and hypokalemia-induced early afterdepolarizations (EADs) in rabbit ventricular myocytes. A, No EADs arose under control conditions at PCL 6, 1, or 6 s in the presence of 4-AP (2 mmol/L). B and C, After exposure to \( \text{H}_2\text{O}_2 \) (1 mmol/L; \( \text{B} \)) or hypokalemia (2.7 mmol/L; \( \text{C} \)), 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). Simultaneous action potentials under the 3 conditions are shown below.
is not completely selective for $I_{\text{to}}$ such that 4-AP off-target effects could have also been responsible.

$I_{\text{to}}$ Reconstitution Reverses EAD Suppression by 4-AP

To determine whether EAD suppression by 4-AP was related primarily to its blockade of $I_{\text{to}}$ rather than its off-target effects, we injected a virtual dynamic-clamp $I_{\text{to}}$ resembling the native rabbit $I_{\text{to}}$. 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 $I_{\text{to}}$ for any parameter combinations tested (46 trials, 6 myocytes, 5 rabbits). Thus, when repolarization reserve was normal, $I_{\text{to}}$ reconstitution did not promote EADs de novo.

However, after EADs had already been induced by either $\text{H}_2\text{O}_2$ or hypokalemia and subsequently suppressed by 4-AP, injection of a virtual $I_{\text{to}}$ with properties approximating the native rabbit $I_{\text{to}}$ (Table 1) caused EADs to reappear (representative illustration in Figure 2, row 4). For the same value of $G_{\text{to}}$, increasing the pedestal current from 0% to 50% or 55% of peak $I_{\text{to}}$ caused EADs to disappear and APD to shorten markedly (Figure 2, row 5). Likewise, increasing $G_{\text{to}}$ without increasing the pedestal had the same effect (Figure 2, row 6).

$I_{\text{to}}$ 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 $I_{\text{to}}$ (chiefly $I_{\text{to1,s}}$) has an unusually long time constant of recovery from inactivation, averaging 6 s at −80 mV. Thus, whereas $I_{\text{to1,s}}$ amplitude is substantial at a PCL of 6 s because of the long diastolic recovery interval between beats, $I_{\text{to1,s}}$ 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 virtual \( I_{\text{to}} \) programmed with instantaneous recovery from inactivation kinetics at \(-80\) mV during PCL 1 s to determine whether EADs reappear, and, if so, to evaluate what properties of \( I_{\text{to}} \) are required.

To assess the independent contribution of each of the 3 \( I_{\text{to}} \) parameters to EAD formation, we introduced a virtual \( I_{\text{to}} \), varying only 1 parameter at a time (Figures 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.** Effects of \( \tau_{\text{inact}} \) on reappearance of pacing-suppressed early afterdepolarizations (EADs). Row 1: EADs induced by \( \text{H}_2\text{O}_2 \) (1 mmol/L) at pacing cycle length (PCL) 6 s (not shown) were suppressed by shortening PCL to 1 s. Rows 2 to 4: A virtual \( I_{\text{to}} \) with \( G_{\text{to}}=0.05 \) 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 4.** Effects of \( \tau_{\text{inact}} \) on reappearance of pacing-suppressed early afterdepolarizations (EADs). Row 1: EADs induced by \( \text{H}_2\text{O}_2 \) (1 mmol/L) at pacing cycle length (PCL) 6 s (not shown) were suppressed by shortening PCL to 1 s. Rows 2 to 4: A virtual \( I_{\text{to}} \) with \( G_{\text{to}}=0.05 \) 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 early afterdepolarizations (EADs). Row 1: EADs induced by \( \text{H}_2\text{O}_2 \) (1 mmol/L) at pacing cycle length (PCL) 6 s (not shown) were suppressed by shortening PCL to 1 s. Rows 2 to 6: A virtual \( I_{\text{to}} \) with \( G_{\text{to}}=0.025 \) nS/pF and \( \tau_{\text{inact}}=80 \) ms reconstituted EADs (*) for pedestals  ≤ 50% (rows 2–5), but not for a pedestal of 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 1113 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, before injecting a virtual \( I_{\text{to}} \). In the plots of \( G_{\text{to}} \) versus \( \tau_{\text{inact}} \) parameter combinations of the virtual \( I_{\text{to}} \) that caused EADs to reappear at PCL 1 s are indicated by solid symbols, whereas those that did not are indicated by open symbols. The 4 plots, labeled A–D, correspond to the size of the pedestal, which was 0% in A, 10% to 24% in B, 25% to 49% in C, and 50% to 75% in D.

**Figure 6A.** A wide range of \( (G_{\text{to}}, \tau_{\text{inact}}) \) parameter combinations (outlined by the dashed black line) caused EADs...
to reappear when the virtual Ito had no pedestal component. The distribution of these parameter combinations agrees well with theoretical predictions from computer modeling simulating the effects of an Ito with 0% pedestal on EAD formation. The gray-shaded area indicates the region in (Gto, \(\tau_{\text{inact}}\)) 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 (\(P<0.0001\); 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–6D reveals the EAD-suppressing effects of pedestal current. As the pedestal component became larger, the region in the (Gto, \(\tau_{\text{inact}}\)) parameter space causing EADs to reappear (outlined by the colored regions) became progressively smaller, consistent with previously reported theoretical predictions. No EADs reemerged with pedestal current >75\%.

Finally, the red box in Figure 6B encloses parameter combinations representative of the human ventricular Ito (predominantly Ito1) reported in the literature for both normal and failing human hearts. This region falls within the virtual Ito parameter region causing EADs to re-emerge.

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

Table 2. Statistical Analysis of Predicted vs Observed (Gto, \(\tau_{\text{inact}}\)) Parameter Combinations Causing EADs to Reappear

<table>
<thead>
<tr>
<th>No. of Gto–(\tau_{\text{inact}}) Combinations</th>
<th>EAD(^+)</th>
<th>EAD(^-)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted</td>
<td>235</td>
<td>0</td>
<td>480</td>
</tr>
<tr>
<td>EAD(^+)</td>
<td>186</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>EAD(^-)</td>
<td>22</td>
<td>223</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>208</td>
<td>272</td>
<td></td>
</tr>
</tbody>
</table>

Positive predictive value [95\% CI]

| 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] |
| PValue                  | <0.0001 |

A total of 480 observed (Gto, \(\tau_{\text{inact}}\)) combinations of a virtual Ito with no pedestal (787 trials in 79 ventricular myocytes from 25 rabbit hearts) that did (EAD\(^+\)) or did not (EAD\(^-\)) cause H2O2-induced or hypokalemia-induced EADs to reappear at pacing cycle length (PCL) 1 s are compared with theoretical predictions from a computer model. CI indicates confidence interval; and EAD, early afterdepolarization.
I\textsubscript{to} as Friend and Foe in EAD Genesis

Transient outward currents have been reported to both suppress and promote EADs.\textsuperscript{2,3} Because of the wide diversity of I\textsubscript{to} properties between different subtypes (I\textsubscript{to1,s}, I\textsubscript{to1,f}, and I\textsubscript{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\textsubscript{to} properties affect EAD formation in diverse experimental settings. Moreover, the previous experimental evidence that I\textsubscript{to} promotes EADs has relied solely on the disappearance of EADs after applying 4-AP to block I\textsubscript{to}. However, 4-AP is not completely selective for I\textsubscript{to} and has significant off-target effects on other ionic currents.\textsuperscript{31,32} The dynamic clamp allows the effects of I\textsubscript{to} on EADs to be tested directly without this complication.

The role of I\textsubscript{to} in EAD formation is a critical issue to understand because derangements in I\textsubscript{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\textsubscript{to} blockade and activation have been suggested as potential antiarrhythmic strategies.\textsuperscript{2,3} Downregulation of I\textsubscript{to} contributes to reduced repolarization reserve in heart failure,\textsuperscript{33} which has been linked to higher risk of triggered ventricular arrhythmias.\textsuperscript{34} Genetic ablation of I\textsubscript{to} in transgenic mice by Kv1.4\textsuperscript{−/−} and Kv4.2W362F crossbreeding\textsuperscript{35} or KChIP2 knockout\textsuperscript{36} significantly prolonged APD, promoting EADs in single ventricular myocytes\textsuperscript{37} and markedly prolonged QT interval, promoting spontaneous ventricular tachycardia in intact tissue in the absence of ventricular hypertrophy or heart failure.\textsuperscript{38} Potentiation of I\textsubscript{to} to increase repolarization reserve has therefore been suggested as a potential antiarrhythmic strategy in heart failure, with the caveat that excessive I\textsubscript{to} can cause arrhythmias by a different mechanism, namely phase 2 re-entry as in Brugada syndrome\textsuperscript{39} and acute ischemia.\textsuperscript{40}

Our findings indicate that additional caution is warranted because in addition to these potential proarrhythmic effects of excessive I\textsubscript{to}, even mild to moderate augmentation of I\textsubscript{to} may be proarrhythmic by promoting EADs when repolarization reserve is already compromised. However, we emphasize that when repolarization reserve was normal, adding a virtual I\textsubscript{to} to the normal rabbit ventricular AP never induced EADs. Hence, for I\textsubscript{to} 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 I\textsubscript{to} is an absolute requirement for EADs. Rather I\textsubscript{to}, in 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 I\textsubscript{to} properties promoting EADs in this study also provides novel insights into the apparent discrepancy between the study of Zhao et al,\textsuperscript{3} which concluded that I\textsubscript{to} promotes EADs in ventricular and Purkinje myocytes from multiple species, and the dynamic clamp study of Workman et al,\textsuperscript{2} which concluded that I\textsubscript{to} suppresses EADs in rabbit and human atrial myocytes. Atrial tissue has a large endogenous I\textsubscript{to} that accounts for the triangular shape of its AP. This large I\textsubscript{to} is in the range that typically suppresses EADs (>0.10–0.15 nS/pF in Figure 6), such that reducing I\textsubscript{to} by dynamic clamp subtraction brought G\textsubscript{to} into the range that frequently promotes EADs (<0.10 nS/pF). Indeed, in the study of Workman et al,\textsuperscript{2} G\textsubscript{to} averaged 0.34 nS/pF in rabbit atrial myocytes and 0.12 nS/pF in human atrial myocytes. In contrast, ventricular myocytes from most nonrodent mammals have a smaller I\textsubscript{to} density (Table 1) that may place them in the range of G\textsubscript{to}, that facilitates EAD formation such that I\textsubscript{to} block with 4-AP or other agents will suppress EADs. Rat and mouse ventricular myocytes, however, have higher I\textsubscript{to} densities than larger mammals, but may also develop EADs that are suppressed by 4-AP,\textsuperscript{3} suggesting that other differences between ventricular and atrial electrophysiology may also be important.

Applicability to Human I\textsubscript{to}

Our study is the first systematic analysis demonstrating that the range of I\textsubscript{to} properties capable of promoting EADs is wide and inclusive of I\textsubscript{to} from other species than just rabbit. We show that EADs were promoted over a 30-fold range of I\textsubscript{to} conductance, favored by an inactivation time constant τ\textsubscript{inact} ≥20 ms, and a pedestal component <25% of peak I\textsubscript{to}. This range includes the typical I\textsubscript{to1} characteristics of both healthy and failing human ventricles, which exhibited a single inactivation time constant τ\textsubscript{inact}, averaging 8 to 75 ms and a pedestal of 16% to 22% in normal and failing epicardial ventricular myocytes.\textsuperscript{10,12,40} 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\textsubscript{to} occurs not just when EADs are induced by oxidative stress with H\textsubscript{2}O\textsubscript{2} (which significantly modified I\textsubscript{to} properties),\textsuperscript{3} 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\textsubscript{2}O\textsubscript{2} augments the late Na current and Ca currents primarily through oxidative CaMKII (Ca/calmodulin-dependent protein kinase II) activation (reflected in the more common appearance of delayed afterdepolarizations in association with H\textsubscript{2}O\textsubscript{2}-induced EADs, rather than with hypokalemia-induced EADs, as in Figure 2).\textsuperscript{41,42} Thus, the specific mechanism by which overall repolarization reserve is reduced does not seem to be critical to the ability of I\textsubscript{to} to promote EADs. The overall implication is that I\textsubscript{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\textsubscript{to}

The mechanism by which I\textsubscript{to} potentiates EADs is consistent with the dynamic theory of EAD formation by a Hopf-homoclinic bifurcation mechanism.\textsuperscript{43} In this theory, EADs are generated by the opposing effects of inward I\textsubscript{CaL}, which is activated as the plateau voltage dips below 0 mV and outward K currents, particularly I\textsubscript{Ks}, which is reactivated during the I\textsubscript{CaL}-mediated EAD upstroke. The activation–deactivation kinetics of I\textsubscript{to} must be matched appropriately to I\textsubscript{Ks} recovery kinetics to achieve membrane potential oscillations,\textsuperscript{44} the defining feature of EADs. If I\textsubscript{to} activates too rapidly, then
repolarization rate is too fast for $I_{\text{CaL}}$ to reactivate and prevent repolarization. $I_{\text{o}}$ can promote EADs by lowering the voltage during the early plateau, thereby slowing $I_{\text{Ks}}$ activation (because $I_{\text{o}}$ activation rate and its open probability are highly voltage dependent) while giving $I_{\text{CaL}}$ enough time to reactivate and oppose full repolarization. Thus, although $I_{\text{o}}$ always increases early repolarization reserve, the indirect effect of $I_{\text{o}}$ on the temporal evolution of other voltage-dependent currents such as $I_{\text{Ks}}$ and $I_{\text{CaL}}$ can paradoxically reduce late repolarization reserve, which is the critical phase during which EADs develop. This also explains why a larger $I_{\text{o}}$ pedestal current tends to suppress EADs because the outward pedestal current directly increases late repolarization reserve, compensating for the reduction in $I_{\text{Ks}}$.

The agreement in Figure 6A between the computer model and the experimental findings lends further support to the dynamic theory of EAD formation via $I_{\text{CaL}}$ reactivation, although other factors such as Ca cycling dynamics may also contribute importantly to EAD formation in many settings.\(^44\) 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 $I_{\text{o}}$ 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 $I_{\text{o}}$-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 AP plateau, thus can further directly reduce late repolarization reserve.

**Study Limitations**

To keep the number of parameters manageable, we simplified the virtual $I_{\text{o}}$ formulation to include only a single inactivation time constant $\tau_{\text{max}}$, whereas 2 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 noninactivating 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 $I_{\text{o}}$ pedestal current.

We did not explicitly test scenarios in which multiple virtual $I_{\text{o}}$ currents with different parameter combinations were added together into the same myocyte, although multiple $I_{\text{o}}$ subtypes ($I_{\text{o1}}, I_{\text{o1}^*},$ and $I_{\text{o2}}$) 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 $I_{\text{o1}}, I_{\text{o1}^*}$ is not a K current, but a Ca-activated Cl current with time course that parallels the intracellular Ca transient.\(^45,46\) Nevertheless, because $I_{\text{o}}$ activates rapidly and inactivates within 20 to 50 ms,\(^45,46\) its kinetics as an outward current fall within the range of virtual $I_{\text{o}}$ 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 $I_{\text{o2}}$, and likewise are expected to fall within the parameter ranges that we tested. Given these limitations, however, our virtual $I_{\text{o}}$ model with 3 independently adjustable parameters should be viewed as a rough guideline for identifying the key EAD-promoting characteristics of $I_{\text{o}}$-like currents.

Although the ranges of virtual $I_{\text{o}}$ parameters in this study encompasses the endogenous $I_{\text{o}}$ parameter ranges from multiple species, the caveat is that we injected the virtual $I_{\text{o}}$ 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 think that the differences would likely be quantitative rather than qualitative given that Zhao et al\(^\text{10}\) found that $I_{\text{o}}$ block with 4-AP suppressed $\text{H}_2\text{O}_2$-induced EADs in multiple species exhibiting markedly different AP properties.

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**Disclosures**

None.

**References**


SUPPLEMENTAL MATERIAL

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.1 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:

\[ I_{to} = G_{to} x_{tof} (\alpha + (1-\alpha) y_{tof})(V - E_k) \]  
\[ \tau_{ytolf} = \tau_{inact} \cdot \left( \frac{1.0}{\left( 1.0 + e^{\frac{V+33.5}{10.0}} \right) + 1.0} \right) \]  

(Eq.1)

(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 $\tau_{ytolf}$ by varying $\tau_{inact}$ (Eq.2).

**Computational modeling**

To generate EADs in our computer simulations, we simulated the H$_2$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 $I_{CaL}$ after H$_2$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 H$_2$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\(_{\text{to}}\) trajectories. (A) Virtual I\(_{\text{to}}\) currents (red traces) with \(\tau_{\text{inact}} = 250\) ms, pedestal of 25%, and variable \(\bar{G}_{\text{to}}\) 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\(_{\text{to}}\) currents with different \(\bar{G}_{\text{to}}\) values from experiments illustrated in (A).
SUPPLEMENTAL REFERENCES


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