Probing cellular arrhythmogenesis using the O'Hara-Rudy model of the undiseased human ventricular cardiomyocyte

Gianluca Selvaggio, Hongbin, Wan, Robert A. Pearlstein *

Global Discovery Chemistry, Computer-Aided Drug Discovery, Novartis Institutes for BioMedical Research, 181 Massachusetts Avenue, Cambridge, MA 02139, USA.

*Corresponding authors

E-mail: robert.pearlstein@novartis.com

Abstract

The ventricular action potential (AP) is subserved by an interdependent system of voltage-gated ion channels and pumps that both alter and respond (directly or indirectly) to the dynamic transmembrane potential ($\Delta\psi_m(t)$) via voltage-dependent state transitions governing inward and outward ion currents. The native dynamic inward-outward current balance is subject to disruption caused by acquired or inherited loss or gain of function in one or more ion channels or pumps. Building on our previous work, we used a modified version of the O'Hara-Rudy (ORd) model of the undiseased human ventricular cardiomyocyte to study the pro-arrhythmic effects of three types of arrhythmia-inducing perturbations in midmyocytes (M cells):

- 1) Blockade of the human ether-a-go-go related gene (hERG) K^+ channel introduced via a Markov state binding model.
- 2) Mutation-induced voltage shifts in hERG channel gating, resulting in faster inactivation or slowed recovery of both phosphorylated and non-phosphorylated forms of the channel (known as LQT2 syndrome).
- 3) Mutation-induced voltage shifts in $Na_v1.5$ gating, resulting in slowed late inactivation of the phosphorylated and non-phosphorylated forms of the channel (known as LQT3 syndrome).

We studied the relationships between ion current anomalies and AP morphology as a function of cycle length (CL) and perturbation type/level. The results are summarized as follows:

- 1) AP duration (APD) is governed directly by $Kir2.1$ activation (I_{K1}), which is delayed when repolarization is slowed by abnormal net inward tipping of the dynamic inward-outward

current balance (reflected in decreased $d(\Delta\psi_m(t))/dt$ during the late AP repolarization phase). In the case of hERG blockade by non-trappable compounds, the perturbation level consists of the dynamic fractional occupancy of the channel, which is governed by blocker k_{on} relative to the rate of channel opening, pharmacokinetic exposure, and k_{off} (in that order).

- 2) Arrhythmia progresses from prolonged paced APs $\rightarrow$ atypical APs (spontaneous and paced) $\rightarrow$ self-sustaining oscillations. Abrupt transitions between these regimes occur at CL- and perturbation-specific thresholds (denoted as T_1 , T_2 , and T_3 , respectively), whereas intra-regime progression proceeds in a graded fashion toward the subsequent threshold. APD and $d(\Delta\psi_m(t))/dt$ during the late repolarization phase varied significantly across the 200 APs of our simulations near the T_1 threshold at CL = 1/35 min, reflecting increasing instability of the AP generation system.
- 3) Arrhythmic APs exhibit highly variable cycle-to-cycle morphologies, depending on the perturbation level, type, and phasing between the underlying ion channel states and pacing cycle.
- 4) Atypical APs may be triggered by typical or atypical depolarizations prior to the T_3 threshold, depending on perturbation type/level and phasing relative to CL:
 - a) APD/CL resides outside of the Goldilocks zone:
 - i) APD/CL $\rightarrow$ 1 at shorter CL and/or longer APD, resulting in pro-arrhythmic “collisions” between successive paced APs (AP_i and AP_j) within a given

cardiomyocyte. We studied this scenario at 60 and 80 beats per minute (BPM), equating to $CL = 1/60$ and $1/80$ min.

ii) $APD/CL < 1$ at longer CL results in spontaneous atypical depolarizations within prolonged paced APs at elevated takeoff $\Delta\psi_m(t)$ and increased channel phosphorylation levels. We studied this scenario at $CL = 1/35$ min.

- b) APD and $d(\Delta\psi_m(t))/dt$ during the late repolarization phase become increasingly variable over successive APs on approach to the T_1 threshold, which is the possible source of short-long-short sequences observed in the ECG preceding torsades de pointes arrhythmia (TdP).
- 5) All atypical depolarizations are solely $Ca_v1.2$ ($I_{Ca,L}$)-driven ($\Delta\psi_m(t)$ falls within the $Na_v1.5$ inactivation window), whereas typical depolarizations are $Na_v1.5$ (I_{Na}) + $I_{Ca,L}$ -driven. Atypical depolarization versus typical repolarization occurrences are determined by the faster of $Ca_v1.2$ and $Kir2.1$ (I_{K1}) activation (where I_{K1} becomes increasingly dampened as the minimum $\Delta\psi_m(t)$ drifts above the $Kir2.1$ activation window).
- 6) $Ca_v1.2$ inactivation gates reset to the open position (accompanied by recovery) synchronously with channel closing under control conditions, generating a small $I_{Ca,L}$ window current in the process. This current grows toward a depolarizing spike when the lag time between recovery and closing grows above a threshold level.
- 7) APs undergo damped oscillatory $Ca_v1.2$ recovery/re-inactivation cycles above the T_3 threshold, which are refreshed by subsequent pacing signals (nodal or reentrant in origin).

Introduction

As for all cellular systems, the AP generation systems of excitable cells, including cardiomyocytes, operate within the non-equilibrium/non-linear dynamics regime [1]. Stable APs depend on tight dynamic counter-balancing between inward and outward ion currents, such that $\Delta\psi_m(t)$ neither under- nor overshoots into the proarrhythmic range. Atypical APs, referred to as early afterdepolarizations (EADs), result from inward-outward ion current imbalances caused by acquired or inherited loss or gain of function in outwardly or inwardly conducting voltage-gated ion channels (notably hERG, KCNQ1, and Kir2.1 versus $\text{Na}_v1.5$ and $\text{Ca}_v1.2$) or pumps. EADs may progress further to self-sustaining cellular and organ level tachyarrhythmias (denoted as CAs and OAs, respectively), including TdP [2–5]. The molecular causes of CA and OA include:

- 1) Prolongation of AP duration (APD) in inherited long QT syndromes (manifesting as chronic QT prolongation in the ECG) caused by mutations in hERG (LQT2), $\text{Na}_v1.5$ (LQT3), or $\text{Ca}_v1.2$ channels (LQT8).
- 2) Loss of outward Kir2.1 current (I_{K1}) [6], accompanied by gain of ryanodine receptor (RyR_2)-mediated Ca^{2+} release [7] in heart failure, resulting in delayed afterdepolarizations (DADs) and ventricular fibrillation [2,3].
- 3) Gains in $\text{Ca}_v1.2$ current ($\text{I}_{\text{Ca,L}}$), late $\text{Na}_v1.5$ current ($\text{I}_{\text{Na(late)}}$), or the Ca^{2+} transient, combined with reduced repolarizing K^+ currents, resulting in APD prolongation and EADs in human hypertrophic cardiomyopathy (HCM) [8,9].
- 4) Loss of I_{K1} and increased Ca^{2+} transient, resulting in rabbit tachyarrhythmia (suggesting a synergistic relationship between these factors) [10].

Understanding the detailed mechanisms by which atypical APs arise is critical for drug safety assessment and the development of therapies targeted at LQT syndromes. Ion channel and electrical dysfunction have been well studied experimentally in the *in vitro*, *ex vivo*, and *in vivo* settings using electrophysiology (e.g. patch clamp) and biochemical approaches, monophasic action potential (AP) recordings in isolated hearts and tissue preparations, and electrocardiography, respectively. However, studies at the cellular level have been hampered by the lack of stable human myocardial cell lines that fully recapitulate normal and abnormal APs under *in vivo*-like conditions, resulting in the need for high quality *in silico* surrogates such as the O'Hara-Rudy (ORd) simulation of the undiseased human ventricular (endo-, epi-, and midmyo) cardiomyocyte [11]. In our previous work, we used a modified version of the ORd model (denoted as ORd/hERG Markov [12]) to simulate the dynamic state distribution of the hERG potassium channel, and characterize the relationships between blocker binding kinetics (BK), dynamic hERG blocker occupancy, and EADs [12] (noting that CA is an emergent, rather than programmed, behavior of this model). We later used ORd/hERG Markov to study the mechanisms of positive and negative hERG channel potentiation [13]. Here, we again use this model to investigate the detailed mechanisms of arrhythmogenesis in M cells under conditions of 1) hERG channel blockade; 2) LQT2 syndrome, which we simulated by speeding and slowing hERG inactivation and recovery, respectively; and 3) LQT3 syndrome, which we simulated by slowing inactivation of late $\text{Na}_v1.5$ current (denoted as $I_{\text{Na}(\text{late})}$). We set about to characterize the arrhythmogenesis process as a function of perturbation type and level, focusing on:

- 1) The chronic reversible sub-arrhythmic regime, as exists in the at-risk patient population, versus the acute arrhythmic regime (the point of no return).
- 2) Perturbation-induced alterations in ion currents, voltage, Ca^{2+} , and other arrhythmogenic factors.
- 3) Perturbation severity-response relationships, including hERG blocker dose-response, and ion channel gain or loss of inactivation function-response relationships.

Materials and methods

We previously generated the ORd/hERG Markov model via substitution of the original Hodgkin-Huxley-based hERG formulation with a published Markov formulation ([14] and Figure 3 of [12]). All ORd/hERG Markov simulations were performed in MATLAB™ 2017b (The MathWorks Inc., Natick, MA). The major components of the AP-generation system considered in this work are shown in Figure 1 (adapted from [11]).

Figure 1. The major channels/pumps/currents considered in this work. Ca^{2+} , Na^{+} , and K^{+} , which are maintained in dynamic non-equilibrium distributions by ion pumps/exchangers, translocate down their electrochemical gradients (toward equilibrium) in response to ion channel activation. Inward and outward transmembrane Ca^{2+} and Na^{+} fluxes occur via voltage-gated channels and intracellular and subspace NCX exchangers (in which the outward Ca^{2+} flux is driven by the transmembrane Na^{+} gradient). Free Ca^{2+} entry/exit to/from the sarcoplasmic reticulum (SR) is driven by the trans-SR concentration gradient. The rates of entry, intra-SR translocation, and exit (J_{up} , J_{tr} , and J_{rel}) are governed by free energy barriers.

hERG blockade

As in our previous work [12], we used a non-trappable hERG blocker exhibiting $k_{\text{on}} = 10^8 \text{ M}^{-1}\text{s}^{-1}$, $k_{\text{off}} = 2.0 \text{ s}^{-1}$, and $\text{IC}_{50} = 20 \text{ nM}$ to probe the arrhythmogenesis mechanism in M cells (chosen based on the lower hERG density and concomitantly greater sensitivity of these cells to perturbations compared with their epi- and endocardial counterparts [12]). We calculated the constant fractional occupancy of trappable blockers based on the open + inactivated + closed state populations, and the dynamic fractional occupancy of non-trappable blockers from the occupied open + inactivated states relative to the total open + inactivated state populations. We then used the putative pro-arrhythmic criteria derived from our study to characterize hERG blocker dose-response relationships for several BK profiles at cycle length (CL) = 1/35, 1/60, and 1/80 min. The hERG IC_{50} was maintained in all cases at $1.0 \mu\text{M}$, the typical upper practical limit of hERG potency vis-a-vis the Redfern safety index (SI) [15] (noting that the upper therapeutic free C_{max} in humans at this IC_{50} is constrained by the Redfern SI to $\sim 33 \text{ nM}$).

We developed an automated algorithm to identify the BK and CL-dependent hERG blocker concentrations at which APs transition from the pre-arrhythmic regime (denoted as the PT_1

threshold) to the arrhythmic regime (denoted as the T_1 threshold), which is approached asymptotically as a function of fractional decrements in blocker concentration (loosely analogous to the equivalence point in acid-base titrations). The algorithm consists of ORd/hERG Markov simulations performed over a series of blocker concentrations, beginning with a user-specified initial guess ($C_i < PT_1$), which was then incremented in steps (denoted as s) of $0.1 \cdot C_i$ until the T_1 threshold is crossed, at which point the concentration was reset to the prior sub- T_1 concentration, the step size reduced by $s/4.0$, and the process repeated. We set the convergence criterion to $s < 0.001$, combined with the absence of atypical depolarizations during each simulation.

LQT2

We simulated pro-arrhythmia and arrhythmia associated with loss of the hERG current (denoted as I_{Kr}) by speeding hERG inactivation and slowing recovery from inactivation, as follows:

Inactivated $\rightarrow$ open state transition (denoted as α_i in the hERG Markov model) (Figure 2A):

$$\alpha_i = 5.45 \cdot 10^{-1} \cdot \exp(-8.17 \cdot 10^{-1} \cdot (V + V_{shift(\alpha_i)}) \cdot F/(R \cdot T)) \cdot 4.5/k_o \quad (1)$$

where V is the transmembrane voltage, $V_{shift(\alpha_i)}$ is a voltage shift factor used to slow this state transition, F is the Faraday constant, R is the gas constant, T is the temperature, and k_o is the extracellular K^+ concentration.

Open $\rightarrow$ inactivated state transition (denoted as β_i in the hERG Markov model) (Figure 2B):

$$\beta_i = 8.2 \cdot 10^{-1} \cdot \exp(5.04 \cdot 10^{-1} \cdot (V + V_{shift(\beta_i)}) \cdot F/(R \cdot T)) \cdot (4.5/k_o)^3 \quad (2)$$

where $V_{shift(\beta_i)}$ is a voltage shift factor used to speed this state transition. We then studied arrhythmogenesis as a function of increasing $V_{shift(\alpha_i)}$ and $V_{shift(\beta_i)}$.

A

B

Figure 2. (A) Plot of the voltage dependence of the hERG open $\rightarrow$ inactivated state transition (α_i) with $V_{shift(a_i)} = 0$. The channel population is strongly inactivated during depolarization. (B). Plot of the voltage dependence of the hERG inactivated $\rightarrow$ open state transition (recovery from inactivation) (β_i) with $V_{shift(\beta_i)} = 0$. The channel population undergoes rapid recovery during repolarization (plots of these state transitions are provided in Figure 1 of [12]).

LQT3

We simulated pro-arrhythmia and arrhythmia associated with increased $I_{Na(late)}$ by slowing the late inactivation gate of $Na_v1.5$ (denoted as h_L), which is calculated in the ORd and ORd hERG/Markov models via integration of:

$$\frac{dh_L}{dt} = (h_{L,ss} - h_L)/\tau_{h,L} \quad (3)$$

where $h_{L,ss}$ is the voltage-dependent term (Figure 3A), and $\tau_{h,L}$ is the gating time step = 200 ms. A voltage shift term ($V_{shift} \leq 0$) introduced to $h_{L,ss}$ was used to slow inactivation, as follows (Figure 3B):

Non-phosphorylated:

$$h_{L,ss} = 1.0/(1.0 + \exp((V + 87.61 + V_{shift})/7.488)) \quad (4)$$

Phosphorylated:

$$h_{L,ss} = 1.0/(1.0 + \exp((V + 93.81 + V_{shift})/7.488)) \quad (5)$$

We then studied arrhythmogenesis as a function of increasing $|V_{shift}|$ (the modified equation 3 is exemplified in Figure 3C for $V_{shift} = -74.1893$ mV).

A

B

C

Figure 3. (A) Plot of $h_{L,ss}$ for the wild type non-phosphorylated $Na_v1.5$ population. Late inactivation occurs at voltages ≥ -40 mV, reaching ~50% maximal inactivation at -90 mV (noting $I_{Na(late)}$ results from partial inactivation of the channel population). (B) Plot of $h_{L,ss}$ for the mutant non-phosphorylated channel population, exemplified by an arbitrary $V_{shift} = -80$ mV, which results in late $Na_v1.5$ inactivation at voltages $\geq +30$ mV, reaching zero inactivation prior to closing at voltages ≤ -50 mV. (C) Upper panel: The simulated h_L gate for wild type ($V_{shift} = 0.0$) and mutant (exemplified with $V_{shift} = -74.1893$ mV) channels (orange and light blue tracings, respectively), noting a decrease in overall channel inactivation throughout the AP. Lower panel: APs corresponding to control and perturbed conditions (orange and light blue tracings, respectively).

Ion current knockouts/knockdowns

I_{Kr} and I_{Kl} (activated during phases 0 and 3-4 [16], denoted as $I_{Kl(early)}$ and $I_{Kl(late)}$, respectively) knockouts were generated by fixing their values at 0. CaMKII knockdown was generated by fixing the K_M (KmCaMK) to 10.0 (default = 0.15).

Isolation of individual contributions to $I_{Ca,L}$ and I_{Na}

$Ca_v1.2$ and $Na_v1.5$ currents were calculated separately for each of their individual activation and inactivation mechanisms based on the following expressions taken from [17]:

Calculation of $I_{Ca,L}$

Activation:

$$\text{Non-phosphorylated: } I_{Ca,L,act} = \overline{I_{Ca,L}} \cdot d \cdot (1 - \phi_{Ca,L}) \quad (6)$$

$$\text{Phosphorylated: } I_{Ca,L,act}^p = \overline{I_{Ca,L}} \cdot d \quad (7)$$

Inactivation:

$$I_{Ca,L_{inact}} = \overline{I_{Ca,L}} \cdot d \cdot (1 - \phi_{Ca,L}) \cdot (f \cdot (1 - n) + f_{Ca} \cdot n \cdot j_{Ca}) + \overline{I_{Ca,L}^p} \cdot d \cdot \phi_{Ca,L} \cdot (f^p \cdot (1 - n) + f_{Ca}^p \cdot n \cdot j_{Ca}) \quad (8)$$

where $\overline{I_{Ca,L}}$ and $\overline{I_{Ca,L}^p}$ are the maximum currents through non-phosphorylated and phosphorylated $Ca_v1.2$, respectively; d is the rate of channel activation; n and $(1 - n)$ denote the fraction of channels undergoing Ca^{2+} - (CDI) and voltage-dependent inactivation (VDI), respectively; $(1 - \phi_{Ca,L})$ and $\phi_{Ca,L}$ represent the fraction of non-phosphorylated and CaMKII-phosphorylated channels, respectively; j_{Ca} denotes the recovery rate from Ca^{2+} -dependent inactivation; and f and f^p , and f_{Ca} and f_{Ca}^p denote the rates of VDI and CDI for non-phosphorylated and phosphorylated channels, respectively. $Ca_v1.2$ channels exhibit fast and slow dynamics for both VDI and CDI, consisting of the following contributions:

$$f = A_{f,fast} \cdot f_{fast} + A_{f,slow} \cdot f_{slow} \quad (9)$$

$$f_{Ca} = A_{Ca,fast} \cdot f_{Ca,fast} + A_{Ca,slow} \cdot f_{Ca,slow} \quad (10)$$

where $A_{f,fast}$ ($= 0.6$) is the fraction of channels undergoing fast VDI, $A_{f,slow} = 1 - A_{f,fast}$ is the fraction of channels undergoing slow VDI, $A_{Ca,fast}$ ($= 0.3 + (0.6/(1.0 + \exp(V - 10)/10))$) is the fraction of channels undergoing fast CDI, and $A_{Ca,slow} = 1 - A_{Ca,fast}$ is the fraction of channels undergoing slow CDI. $I_{Ca,L}$ is thus separable into k currents ($I_{Ca,L_{inact,k}}$) governed by slow/non-phosphorylated ($I_{V,s}$, $I_{VCa,s}$), fast/non-phosphorylated ($I_{V,f}$, $I_{VCa,f}$), slow/phosphorylated ($I_{V,s}^p$, $I_{VCa,s}^p$), and fast/phosphorylated ($I_{V,f}^p$, $I_{VCa,f}^p$) CDI and VDI contributions (eight in total). As

we show later in this work, anomalous depolarizations are mediated largely by $I_{V,f} + I_{VCa,f} + I_{VCa,f}^p + I_{V,f}^p$ (denoted as $I_{Ca,L(late)}$).

Calculation of peak I_{Na} (denoted as $I_{Na(early)}$)

Activation:

$$\text{Non-phosphorylated: } I_{Na,act} = \overline{G_{Na}} \cdot (V - E_{Na}) \cdot m^3 \cdot (1 - \phi_{Na}) \quad (11)$$

$$\text{Phosphorylated: } I_{Na,act}^p = \overline{G_{Na}} \cdot (V - E_{Na}) \cdot m^3 \quad (12)$$

Inactivation:

$$h = A_{h,fast} \cdot h_{fast} + A_{h,slow} \cdot h_{slow} \quad (13)$$

$$\text{Non-phosphorylated: } I_{Na,inact} = \overline{G_{Na}} \cdot (V - E_{Na}) \cdot m^3 \cdot (1 - \phi_{Na}) \cdot h \cdot j \quad (14)$$

$$\text{Phosphorylated: } I_{Na,inact}^p = \overline{G_{Na}} \cdot (V - E_{Na}) \cdot m^3 \cdot \phi_{Na}^p \cdot h^p \cdot j^p \quad (15)$$

Calculation of $I_{Na(late)}$

Activation:

$$\text{Non-phosphorylated: } I_{Na(late),act} = \overline{G_{Na(late)}} \cdot (V - E_{Na}) \cdot m_L \cdot (1 - \phi_{Na(late)}) \quad (16)$$

$$\text{Phosphorylated: } I_{Na(late),act} = \overline{G_{Na(late)}} \cdot (V - E_{Na}) \cdot m_L \quad (17)$$

Inactivation:

$$I_{Na(late),inact} = \overline{G_{Na,Late}} \cdot (V - E_{Na}) \cdot m_L \cdot \left((1 - \phi_{I_{Na,L}}) \cdot h_L + \phi_{I_{Na,L}} \cdot h_{L,CaMKII} \right) \quad (18)$$

where $\overline{G_{Na,Late}}$ is the maximum conductance of the single channel, m_L is the activation rate, h_L and $h_{L,CaMKII}$ are the rates of $Na_v1.5$ channel inactivation in the non-phosphorylated and phosphorylated forms, respectively, and E_{Na} is the sodium reversal potential.

Calculation of APD and $d(\Delta\psi_m(t))/dt$ at AP phase 3

The start time of each AP (phase 0) was determined by parsing the voltage at three successive time points (t_i, t_{i+1}, t_{i+2}), such that $V(t_{i+2}) > V(t_{i+1}) > V(t_i)$ and $V(t_{i+2}) > 2$ mV. The end time of each AP (phase 4) was determined by parsing the voltage at three successive time points, such that $V(t_{i+2}) < V(t_{i+1}) < V(t_i)$ and $V(t_{i+2}) < 2$ mV. The duration of each AP was calculated by subtracting the start time from the end time.

$d(\Delta\psi_m(t))/dt$ at phase 3 (the late repolarization phase [16]) of each AP was calculated by fitting a line to five successive $V(t)$ points centered on -30 mV using the polyfit function of MATLAB (which returns the slope and intercept). We then extended this line to two additional points at $V(t_{i-3})$ and $V(t_{i+3})$ using the polyfit function, which was overlaid on the corresponding AP using the plot function of MATLAB.

Calculation of the area under the curve (AUC) for $I_{V,f}$, $I_{VCa,f}$, $I_{V,f}^p$, and $I_{VCa,f}^p$

The area under the curve (AUC) was used as a metric of the current-time magnitudes conducted by the four fast VDI and CDI states relative to control. AUCs were calculated for each current using the trapz function of MATLAB (skipping the first 150 time points of each respective AP to isolate the late from the peak contribution).

Results

Although anomalous $I_{Ca,L}$ is the known direct cause of atypical depolarizations in CA, the specific mechanisms by which this current is generated are poorly understood. Our previous explanation is clearly oversimplified and incomplete [12], as are those of others [4]. In this work, we demonstrate that increasing disruption of the native dynamic inward-outward current balance leads to progressive AP alterations across three major perturbation thresholds (denoted as T_1 , T_2 , and

T₃). The ion current anomalies driving such alterations differ between hERG blockade/dysfunction and gain of late Na_v1.5 function, and at short versus long pacing CLs. AP alterations progress in a graded fashion as a function of perturbation severity, entering the chaotic oscillatory regime at T₃ (the severity of which is likewise graded). We attempted to de-convolute the effects of hERG and late Na_v1.5 perturbations on the AP and underlying ion currents, as follows:

- 1) Ca_v1.2 channels close in the wake of inactivation gate resetting/opening in preparation for the subsequent AP, resulting in transient recovery of inactivated sub-populations.
- 2) Perturbations promoting delayed Ca_v1.2 closing lead to anomalous retention of the fast-VDI and CDI states, recovery from which evokes an anomalous I_{Ca,L(late)} window current at the T₁ tipping point (comprised principally of $I_{VCa,f}$ and $I_{V,f}$).
- 3) Atypical I_{Ca,L} spikes (~2-fold larger than control) arise when $I_{VCa,f}$, $I_{V,f}$, and subspace Ca²⁺ grow to the tipping point at which CDI and VDI states recover.
- 4) Decreased slope of the AP (i.e. $d(\Delta\psi_m(t))/dt$) during phase 3 (centered at ~ -30 mV) under perturbed conditions results in slowed ion channel gating, including slowed opening of the fast CDI gate, slowed I_{K1} activation, and prolonged APD.
- 5) A CL- and perturbation-dependent “race” between spontaneous (non-paced) atypical I_{Ca,L}-driven depolarization, collision with the subsequent paced AP, and I_{K1}-driven repolarization ensues.
- 6) Collisions depend on APD relative to CL (i.e. APD/CL), whereas spontaneous depolarizations depend on $d(\Delta\psi_m(t))/dt$ and non-uniformity thereof across the AP wave train.

- 7) Increased minimum resting $\Delta\psi_m(t)$, which is mirrored by impaired Kir2.1 activation and I_{K1} knockdown during atypical $I_{Ca,L}$ -driven depolarizations.
- 8) Knockdown of $I_{Na(early)}$, $I_{Na(late)}$, and I_{to} at the takeoff potential, which mirror the level of I_{K1} knockdown.

$I_{Ca,L}$ under control conditions ($APD/CL \ll 1$)

As is well appreciated, $I_{Ca,L}$ plays a prominent role in arrhythmogenesis. $I_{Ca,L}$ and other cardiac ion currents can be partitioned into early and late components coinciding with the depolarization and late repolarization phases (0 and 3 [16]) of the AP, respectively. $I_{Ca,L(early)}$ is triggered by d gate opening in response to paced $Ca_v1.2$ activation (Figure 4A), transitioning to $I_{Ca,L(late)}$ as the channel population distributes among the active and fast and slow phosphorylated and non-phosphorylated VDI and CDI states (Figure 4B). CDI inactivation (which is likewise voltage-gated) is stimulated by elevated subspace Ca^{2+} during AP phases 0 to 2. All CDI and VDI inactivation gates (f_{caf}/f_{cas} and f_f/f_s , respectively) open prior to depolarization, close at gate-specific rates during repolarization, and then reopen prior to channel closing in preparation for the subsequent AP. Small window currents are generated during inactivation gate re-opening prior to channel closing, the largest of which consist of $I_{V_{Ca,f}}$ and $I_{V_{s}}$ (Figure 4B). $I_{V_{Ca,f}}$ grows during AP prolongation, and therefore constitutes an Achilles' Heel of the $Ca_v1.2$ channel gating machinery (noting that $I_{V_{s}}$ remains similar to control under pro-arrhythmic conditions).

A

B

Figure 4. (A) The $Ca_v1.2$ activation gate (denoted as d) opens in response to a paced depolarization, and closes during AP phase 3. Left panel: Registration between the AP (lower plot, red tracing), d gate (upper plot, cyan), and $I_{Ca,L,act}$ (upper plot, auburn tracing) simulated at a CL = 1/35 min. The d gate (light blue tracing) opens during depolarization (noting that the downward leg of d in

the model consists of decreasing activation, rather than closing per se). The channels inactivate during repolarization via the slow (fcas) and fast (fcaf) CDI gates, and the slow (fs) and fast non-phosphorylated (ff) and phosphorylated (ffp) VDI gates, giving rise to $I_{Ca,L(late)} = I_{VCa,f}^p + I_{VCa,s} + I_{VCa,s}^p + I_{V,f}^p + I_{V,s} + I_{V,s}^p$. The fraction of channels in the CDI states (ncas) (gold tracing) is shown, together with the d and fcaf gates (cyan and magenta tracings, respectively). Right panel: The inactivation gates re-open (reset) in preparation for the subsequent AP prior to closing (shown for fcaf). (B) Left panel: $I_{Ca,L}$ window current generated by slight recovery into $I_{VCa,f}$ (via the fcaf gate). Right panel: $I_{Ca,L}$ window current generated by slight recovery into $I_{V,s}$ (via the fs gate).

The major stages of hERG dysfunction-induced arrhythmogenesis

The putative stages of arrhythmogenesis caused by hERG blockade and gain of inactivation/loss of recovery function consist of:

- 1) **Pro-arrhythmic/sub-acute** ($< T_1$): Prolongation of paced APs in the $\Delta\psi_m(t) \cong -50$ mV range, resulting in abnormal buildup of $I_{Ca,L}$ toward the depolarization threshold (as outlined above). The resting voltage always extends into the Kir2.1 activation window.
- 2) **Pre-T₁** (PT₁): The highly unstable regime immediately prior to the depolarization threshold, which serves as a common reference for comparing pro-arrhythmic propensity (noting that this regime exists within a tiny perturbation range that is unlikely encountered in the clinical setting).
- 3) **Pre-arrhythmic/sub-acute** ($\geq T_1$): At long CL (i.e. $APD/CL < 1$), $I_{Ca,L}$ builds to the spontaneous (non-paced) depolarizing level prior to collisions between successive APs (denoted as AP_i and AP_j). At short CL (i.e. $APD/CL \rightarrow 1$), $I_{Ca,L}$ builds to the depolarizing

level during prolonged AP_i , which then “collides” with the subsequent paced AP (Figures 5 and 6A) to form AP_j . The resting voltage always extends into the respective windows of hERG, KCNQ1, and Kir2.1 activation.

- 4) **Pre-arrhythmic/severe sub-acute** ($\geq T_2$). AP_j collides with the subsequent paced AP (denoted as AP_k), resulting in bifurcation of AP_j into AP_j' and AP_j'' (Figures 5 and 6A). The resting voltage always extends into the respective windows of hERG, KCNQ1, and Kir2.1 activation (Figure 6B).
- 5) **Arrhythmic/acute** ($\geq T_3$): APs transition to non-paced oscillatory cycles (Figure 6A). The resting voltage always extends into the respective windows of hERG, KCNQ1, and Kir2.1 activation (Figure 6B).
- 6) **Highly arrhythmic/severe acute** ($\gg T_3$): Non-paced oscillatory cycles are either extinguished infrequently or not at all (Figure 6B). The minimum repolarized voltage rarely (if ever) extends into the Kir2.1 activation window.

Figure 5. The effect of AP prolongation on the loss of restitution time, denoted in this work as $1 - \text{APD/CL}$ between two successive APs (AP_i and AP_j). AP_i and AP_j “collide” when $\text{APD/CL} = 1$, which triggers anomalous (though paced) $I_{\text{Ca,L}}$ -driven depolarization of AP_j .

A

B

Figure 6. (A) The putative stages of arrhythmogenesis driven by hERG blockade or gain of hERG inactivation/loss of recovery function. Progression within and between each stage is graded and non-linear with respect to increasing levels of hERG blockade/dysfunction. Progressively greater APD lengthening occurs at ever finer increments of increasing blocker concentration on approach to T_1 . The prolonged AP-generation system is primed for anomalous AP_j -forming depolarization at the T_1 threshold, when: 1) $APD/CL \rightarrow 1$ (resulting in collisions between successive APs); or 2) $APD/CL < 1$ and the slope of $d(\Delta\psi_m(t))/dt = 0$ mV (typically at longer CL). AP_j is primed for additional anomalous depolarizations upon collision with a subsequent AP (denoted as AP_k), forming AP_j' and AP_j'' . (B) Kir2.1 activation follows the graded increase in $\Delta\psi_m(t)$, which ranges between ~ -90 mV (control) and ~ -25 mV.

The AP-generation system responds to increasing levels of perturbation, as follows:

- 1) Slow quasi-linear growth in APD, accompanied by decreasing $d(\Delta\psi_m(t))/dt$ within the shoulder region. T_1 is approached more rapidly for trappable versus non-trappable blockers exhibiting slower k_{on} , consistent with our earlier argument against a one-size-fits-all safety margin [12] (further addressed in [18]).
- 2) Large swings in successive APD (Figure 7) (short-long-short QT sequences are known to precede TdP). Such behavior, reflecting high instability of the system, is attributable to oscillations in $d(\Delta\psi_m(t))/dt$, APD, and ion channel activity on approach to the T_1 tipping point.
- 3) Abrupt crossing of the T_1 tipping point.

Figure 7. Short-long-short-long durations of four successive APs (top left to right, bottom left to right) caused by hERG blockade at the edge of the T_1 tipping point (blocker attributes shown in bold in Table 1) at $CL = 1/80$ min, resulting from fluctuations in $d(\Delta\psi_m(t))/dt$ at the edge of the Kir2.1 activation window. Such behavior reflects high instability of the system.

We studied AP morphology as a function of hERG blocker concentration, titrated in increasingly fine increments toward the AP_i - AP_j ($APD/CL = 1$) and AP_j - AP_k collision thresholds (T_1 and $T_2 = 51.95$ and 51.9511 nM, respectively) (Figure 8).

Figure 8. Snapshots of hERG blockade effects on APs (including $d(\Delta\psi_m(t))/dt$ shown as tangent lines) at the T_1 and T_2 thresholds (simulated at $CL = 1/60$ min), demonstrating graded responses of the AP waveform to incremental changes in perturbation severity (hERG blocker concentrations as noted). Collisions between successive paced APs (AP_i-AP_j and AP_j-AP_k) result in anomalous depolarizations.

Dynamically counter-balanced systems are subject to oscillations when Yin and Yang are coupled in reciprocal out-of-phase relationships (as in the AP generation system), in which inward currents activate outward currents, and vice versa through their opposing effects on $\Delta\psi_m(t)$. The AP is terminated under control conditions when the current balance is tipped strongly in the

hyperpolarized direction by I_{K1} activation (Figure 9A). However, the I_{K1} activation window ($\Delta\psi_m(t) \leq -50$ mV) (Figure 9B) is inaccessible below a threshold of $I_{Kr} + I_{Ks}$ (noting that I_{K1} knockdown in nodal cells and iPSC-derived cardiomyocytes results in sustained AP oscillations). Total knockout of I_{Kr} results in ringing oscillations in the AP waveform (Figure 10).

A

B

Figure 9. (A) Both control (left) and sub- T_1 prolonged (right) APs are terminated by I_{K1} activation (signaling the end of open ion channel states). (B) R_{k1} , the voltage-dependence of channel activation in the ORd and ORd/hERG Markov models, ($R_{k1} = 1.0/(1.0 + \exp(\frac{V+105.8-2.6 \cdot [K_{out}^+]}{9.493}))$), where V is the voltage and $[K_{out}^+] = 5.4$ mM). APD is thus directly determined by the rate of I_{K1} activation, which in turn, is governed by $d(\Delta\psi_m(t))/dt$ during AP phase 3.

Figure 10. The $\Delta\psi_m(t)$ waveform under conditions of total I_{Kr} knockout at $CL = 1/35$ min. Each arrhythmic cycle consists of a paced depolarization, followed by a series of ringing oscillations (noting that the number of oscillations between pacing signals decreases with decreasing CL). The underlying currents driving this process are discussed in a later section.

The role of Ca^{2+} in arrhythmogenesis

As is widely appreciated, the myocardial contraction cycle is driven by the intracellular Ca^{2+} cycle, which in turn is driven by the AP cycle. The Ca^{2+} cycle consists of the following steps:

- 1) Release from the sarcoplasmic reticulum (SR) via RyR_2 channels into the subspace.
- 2) Diffusion from the subspace to the myoplasm, resulting in transient troponin activation.
- 3) Re-uptake to the SR from the myoplasm via the SERCA pump (Figure 1) and reversal of troponin activation.

The driving force for cyclic inter-compartmental ion translocation consists of transmembrane Ca^{2+} , Na^+ , and K^+ , trans-SR Ca^{2+} , and subspace-intracellular Ca^{2+} gradients that are maintained away from their respective equilibrium distributions (as given by the Nernst equation), and undergo partial rundown during translocation (reset by ion exchange pumps). Ca^{2+} directly activates a host

of molecular targets, including troponin, RyR₂, and CaMKII (which phosphorylates ion channels, ion exchangers, RyR₂, and the SERCA pump), and inactivates Ca_v1.2 via Ca²⁺-dependent inactivation. Positive or negative attenuation of the aforementioned ion gradients is highly pro-arrhythmic. Elevated intracellular Ca²⁺ slows ion channel activation and speeds inactivation via enhanced CaMKII-mediated phosphorylation (an overall anti-arrhythmic effect). However, elevated subspace Ca²⁺ promotes the CDI states of Ca_v1.2 (recovery from which drives atypical depolarizations in our simulations). Recovery from both the CDI and VDI states is further enhanced by increased $\overline{I_{Ca,L}}$ at depolarized $\Delta\psi_m(t)$ resulting from elevated subspace Ca²⁺. $\overline{I_{Ca,L}}$ is calculated in the ORd and ORd/hERG Markov models as follows:

$$\overline{I_{Ca,L}} = 4.0 \cdot Vffrt \cdot (cass \cdot \exp(2.0 \cdot Vf rt) - 0.341 \cdot cao) / (\exp(2.0 \cdot Vf rt) - 1.0)$$

where $vffrt = V \cdot \mathcal{F}^2 / (R \cdot T)$ and $Vf rt = V \cdot \mathcal{F} / (R \cdot T)$, $\mathcal{F}$ is the Faraday constant, R is the gas constant, and V is the voltage. Subspace Ca²⁺ is calculated in the ORd and ORd/hERG Markov models, as follows:

$$dcass = B_{cass} \cdot (- (I_{Ca,L} - 2.0 \cdot I_{NaCa_{ss}}) \cdot \frac{A_{cap}}{2.0 \cdot F \cdot v_{ss}} + J_{rel} \cdot \frac{v_{jsr}}{v_{ss}} - J_{diff})$$

where $dcass$ is the derivative of the free subspace Ca²⁺ concentration (solved via numerical integration in the model), B_{cass} is the free fraction of subspace Ca²⁺, $I_{NaCa_{ss}}$ is the subspace Ca²⁺ efflux current, A_{cap} is the capacitive area, v_{ss} is the subspace volume, v_{jsr} is the junctional SR volume, J_{rel} is the rate of RyR₂ release, and J_{diff} is the rate of subspace to myoplasmic Ca²⁺ diffusion. The dynamic subspace Ca²⁺ level is thus coupled recursively to its inputs and outputs. Anomalous subspace Ca²⁺ elevation results in increased $\overline{I_{Ca,L}}$ (translating to increased $I_{Ca,L}$), which, in turn, drives increased Ca²⁺ release. Dynamic subspace and intracellular Ca²⁺, the activated

CaMKII fraction, and the isolated activated $I_{Ca,L,act}$ fraction are plotted in Figure 11A, 11B, and 11C, respectively.

A

B

C

Figure 11. (A) Free subspace and intracellular Ca^{2+} concentrations (mM) (green and red tracings, respectively) and Ca^{2+} -activated CaMKII (light blue tracing) under control conditions at $CL = 1/35$ min. Free subspace Ca^{2+} spikes during AP phase 0, diffuses to the myoplasm, and binds to various proteins, including troponin, calmodulin, and $Ca_v1.2$. (B) $Ca_v1.2$ is phosphorylated and

dephosphorylated during each AP by activated CaMKII (yellow tracing). (C) Phosphorylation slows $\text{Ca}_v1.2$ activation ($I_{\text{Ca,L,act}}^p$ versus $I_{\text{Ca,L,act}}$).

The effects of hERG dysfunction on ion currents in the APD/CL < 1 regime (hERG blocker concentration < T_1 @ CL = 1/35 min)

Next, we studied the ion current profiles during prolonged APs and spontaneous non-paced $I_{\text{Ca,L}}$ -driven depolarizations in the absence of AP collisions at CL = 1/35 min. We titrated the hERG blocker concentration to the approximate T_1 threshold (~46.35 nM) at this CL, and simulated the APs under these and control conditions. The first ~100 APs exhibited prolonged, but relatively uniform $d(\Delta\psi_m(t))/dt$ in the shoulder region, which became increasingly shallower and variable over the latter ~100 APs where atypical $I_{\text{Ca,L}}$ -driven depolarizations occurred.

$I_{\text{Ca,L}}$ effects during the late pro- and early pre-arrhythmic stages

The late pro-arrhythmic stage (approaching T_1) is characterized by anomalously large $I_{V\text{Ca},f}$ and $I_{V,f}$ window currents resulting from:

- 1) Increased dwelling of phosphorylated and non-phosphorylated $\text{Ca}_v1.2$ in the CDI and VDI states prior to I_{K1} -driven channel closing (Figure 12A). Increased dwelling in the CDI states is attributable to abnormal subspace Ca^{2+} levels (Figure 13A-E) and CaMKII activation (Figure 13F).
- 2) Enhanced recovery of $\text{Ca}_v1.2$ channels from the CDI and VDI states prior to delayed channel closing. $I_{V\text{Ca},f}$ and $I_{V,f}$ broadening (corresponding to slowing of the fcaf and ff gates (Figure 12B)) and slowed I_{K1} activation (Figure 14A) are attributable to decreased $d(\Delta\psi_m(t))/dt$ within the prolonged shoulder region.

$I_{V_{Ca,f}}$ and $I_{V,f}$ broadening grows in a graded fashion with increasing perturbation prior to T_1 . A tipping point in $I_{V_{Ca,f}}$ recovery ensues at T_1 , which promotes voltage-dependent recovery and re-inactivation of all CDI and VDI states via opening and subsequent closing of the f_{caf} , f_{cap} , f_{cas} , f_f , f_{fp} , and f_s gates, respectively (manifesting as a spike in $I_{Ca,L(late)}$) (Figures 14A and 14B). The window current magnitudes during recovery from the CDI and VDI states are limited under control conditions by short lag times between inactivation gate opening/resetting and channel closing. $I_{V_{Ca,f}}$ and $I_{V,f}$ grow with decreasing $d(\Delta\psi_m(t))/dt$, corresponding to slowing of the inactivation gates, together with I_{K1} -mediated channel closing. Vulnerability of this mechanism to delayed closing constitutes a major Achilles' Heel of the $Ca_v1.2$ gating system.

A

B

Figure 12. (A) Increased $I_{V_{Ca,f}}$ during prolonged APs that exhibit anomalously shallow $d(\Delta\psi_m(t))/dt$. The fcaf gate slows significantly under perturbed (solid magenta tracing) versus control (dotted magenta tracing) conditions, resulting in prolonged recovery of the fast CDI state (generating $I_{V_{Ca,f}}$). The fraction of channels in the CDI states (nca) is abnormally large under perturbed (solid gold tracing) versus control (dotted gold tracing) conditions due to Ca^{2+} release from the SR during this time period. (B) Recovery of $I_{V_{Ca,f}}$ and $I_{V,s}$ results from $\Delta\psi_m(t)$ -driven fcaf and fs opening. Broadening of these currents results from slowed fcaf and ff opening due to decreased $d(\Delta\psi_m(t))/dt$ within the shoulder region.

A

B

C

D

E

F

Figure 13. (A) Zoomed out view of subspace Ca^{2+} levels under control (auburn tracing) and perturbed (blue tracing) conditions throughout the 200 APs of the simulation. Elevated Ca^{2+} under perturbed conditions (primarily in the subspace) is due to increased Ca^{2+} cycling (RyR₂-mediated SR release, translocation within the SR, and SERCA-mediated SR re-uptake). (B) Zoomed in view

of time-dependent subspace (left) and intracellular (right) Ca^{2+} levels under control and perturbed conditions (orange and blue tracings, respectively). (C) Ca^{2+} release caused by the $I_{\text{Ca,L}}$ window currents stimulates anomalous release of Ca^{2+} from the SR (coral tracing) relative to control (green tracing), which further promotes transitioning to the CDI states. $nca(t)$ is determined by the rate of Ca^{2+} -dependent buildup (α) versus recovery-dependent decay ($nca \cdot jca$) to/from the CDI states ($d(nca(t))/dt$). (D) Co-dependence of $I_{V,x}$ and $I_{V,x}^p$ window current and subspace Ca^{2+} time-dependent behavior. (E) Co-dependence of $I_{VCa,x}$ and $I_{VCa,x}^p$ window current and subspace Ca^{2+} time-dependent behavior. (F) Zoomed out view of activated CaMKII levels under control and perturbed conditions throughout the 200 APs of the simulation (orange and blue tracings, respectively). Increased CaMKII activation results in enhanced ion channel, exchanger, RyR₂, and SERCA phosphorylation.

A

B

C

D

Figure 14. (A) A spontaneous depolarizing $I_{Ca,L}$ -driven spike occurs at a tipping point in $d(\Delta\psi_m(t))/dt$, f_{caf} , and ff slowing, coinciding with one or more oscillations in time-dependent gating behavior. A single oscillation occurs at the T_1 threshold at this perturbation level, although a second $I_{V_{Ca,f}}$ window current appears at the end of the cycle (i.e. the channels are primed for an additional $I_{Ca,L}$ spike at a higher perturbation level, and additional cycles of CDI/VDI inactivation, recovery, and $I_{Ca,L}$ spikes at still higher levels). (B) $I_{Ca,L,act}$ (purple tracing) at paced and spontaneous depolarizations in the AP complex shown in A and B. Reduced counter-balancing between inward and outward currents during atypical depolarizations results in significantly greater $I_{Ca,L}$ (red arrows), compared with paced depolarizations under both perturbed and control conditions. (C) $I_{Ca,L}$ window current-evoked Ca^{2+} release stimulates anomalous SR-mediated Ca^{2+} release (coral tracing) relative to control (green tracing), which further promotes population of the CDI states. $nca(t)$ is determined by the rate of Ca^{2+} -dependent buildup (α) versus recovery-dependent decay ($nca \cdot j_{ca}$) to/from the CDI states ($d(nca(t))/dt$). (D) Left panel: Full recovery

of $\text{Ca}_v1.2$ channels from all CDI and VDI states occurs at a tipping point of f_{caf} and f_f slowing (at which the two gates undergo a single opening-closing-reopening cycle terminated by I_{K1} activation), which underlies anomalous $\text{I}_{\text{Ca,L}}$ -driven depolarization. Right panel: $I_{\text{VCa},f}$ partially recovers during the subsequent prolonged AP.

The overall pro- $\rightarrow$ pre-arrhythmic transitioning process can be summarized as follows:

- 1) The fast and slow $\text{Ca}_v1.2$ inactivation gates reset to the open state in preparation for the subsequent AP. However, channel closing lags behind recovery from the VDI and CDI states due to decreased $d(\Delta\psi_m(t))/dt$, thereby expanding the $\text{I}_{\text{Ca,L}}$ window current.
- 2) Transitions to/from the CDI states are governed by Ca^{2+} - $\text{Ca}_v1.2$ binding, which under control conditions, cycles between high and low levels during the AP (mirroring subspace Ca^{2+} levels). The Ca^{2+} cycle is disrupted under perturbed conditions, which promotes anomalous Ca^{2+} - $\text{Ca}_v1.2$ binding and transition to the CDI states (Figure 14C).
- 3) The f_{caf} gate slows in concert with the shoulder region of $d(\Delta\psi_m(t))/dt$ under perturbed conditions.
- 4) f_{caf} oscillates at a critical perturbation level (corresponding to a threshold slowing of $d(\Delta\psi_m(t))/dt$), which drives recovery of $I_{\text{VCa},f}$ and $I_{\text{V},f}$, which in turn drive recovery of all other CDI and VDI states. Anomalous $I_{\text{VCa},f}$ is terminated by re-inactivation and channel closing coincident with I_{K1} activation (Figure 14D).
- 5) The f_{caf} oscillation may be extinguished by Kir2.1 activation below the T_2 threshold, or undergo additional recovery/inactivation cycles at or above this threshold (the putative cause of the ringing oscillations observed under I_{Kr} knockout conditions (Figure 10)).

I_{Na} effects during the pro- and pre-arrhythmic stages

All time dependent $Na_v1.5$ gating behaviors are perturbed as follows at hERG blocker concentrations $< PT_1$ (Figure 15):

- 1) Time-dependent accumulation of phosphorylated $Na_v1.5$, mirroring subspace Ca^{2+} levels and Ca^{2+} -mediated CaMKII activation.
- 2) $I_{Na(early)}$ follows the shift from the slower activating $I_{Na,act}$ to the faster activating phosphorylated $I_{Na,act}^p$ fraction across the 200 APs of the simulation (Figure 15A), which is offset by faster inactivation of $I_{Na,fast,inact}^p$ versus $I_{Na,fast,inact}$ (Figure 15B). $I_{Na,slow,inact}$ and $I_{Na,slow,inact}^p$ are weakly altered under perturbed conditions (Figure 15C). As a result, total $I_{Na(early)}$ remains similar to control across the simulation (Figure 15D).

$I_{Na(early)}$ is absent entirely during atypical $I_{Ca,L}$ -driven depolarizations at blocker concentrations $\geq T_1$ (due to inactivation of $Na_v1.5$ channels at the higher takeoff voltage of such depolarizations), whereas h_L is open and $I_{Na(late)}$ is present (Figure 16).

A

B

C

D

Figure 15. (A) $I_{Na,act}$ (left) and $I_{Na,act}^p$ (right) under control (green inset and light orange tracings on the left and right, respectively) and perturbed (purple and dark orange tracings, respectively) conditions. Under perturbed conditions, $I_{Na,act}^p$ grows and $I_{Na,act}$ decreases with increasing time-dependent CaMKII-mediated $Na_v1.5$ phosphorylation. (B) $I_{Na,fast,inact}$ (left) and $I_{Na,fast,inact}^p$ (right) under control (blue inset and purple tracings on the left and right, respectively) and perturbed conditions (auburn and light orange tracings, respectively). These currents likewise mirror increasing time-dependent $Na_v1.5$ phosphorylation. (C) $I_{Na,slow,inact}$ (left) and $I_{Na,slow,inact}^p$ (right) under control (light orange inset and teal blue tracings on the left and right, respectively) and perturbed (dark orange and green tracings, respectively) conditions. As expected, the total $I_{Na(early)}$ (the sum of the phosphorylated and non-phosphorylated contributions) is similar to control (not shown). (D) Total $I_{Na(early)}$ under control (blue tracing) versus perturbed (cyan tracing) conditions are similar due to offsetting between the phosphorylated and non-phosphorylated forms of the channel.

Figure 16. $I_{Na(early)}$ (cyan tracing) is entirely absent during spontaneous $I_{Ca,L}$ -driven depolarizations, whereas $I_{Na(early)}$ (solid magenta tracing, perturbed; dotted magenta tracing, control) is present. $I_{Ca,L}$ (red tracing) and control $I_{Na(early)}$ (auburn tracing) are shown for reference. The $I_{Na(early)}$ inactivation gate (denoted as h) (solid green tracing, perturbed; dotted green tracing, control) is closed during spontaneous $I_{Ca,L}$ -driven depolarizations, whereas the $I_{Na(early)}$ inactivation gate (denoted as hL) (solid blue tracing, perturbed; dotted blue tracing, control) is open.

$I_{Ca,L}$ effects during the extreme arrhythmic stage ($\gg T_3$)

Next, we examined $Ca_v1.2$ inactivation and recovery under total I_{K_r} knockout conditions, in which depolarization and repolarization are entirely $I_{Ca,L}$ - (triggered by paced signals) and I_{K_s} -driven, respectively (noting that very small ion fluxes underlie the abnormally small voltage range of $\Delta\psi_m(t)$). The ringing oscillations shown in Figure 10 are attributable largely to the interplay between $\Delta\psi_m(t)$, the f_{caf} gate (depolarization), I_{K_s} (repolarization) (Figures 17A-B), and exchanger currents (not shown). The oscillations are mirrored by oscillations in subspace Ca^{2+} (Figure 17C).

A

B

C

Figure 17. (A) Total I_{Kr} knockout-induced ringing oscillations in $\Delta\psi_m(t)$ are driven entirely by recovery from the CDI and VDI states of $\text{Ca}_v1.2$ (primarily CDI). (B) Zoomed out view of ringing oscillations in f_{caf} (magenta tracing) and the CDI state populations. (C) Dynamic subspace Ca^{2+} behavior under control (green tracing) and total I_{Kr} knockout conditions.

The effects of hERG dysfunction on ion currents in the APD/CL $\rightarrow$ 1 regime (hERG blocker concentration $\rightarrow T_1$ @ CL = 1/60 min)

Next, we studied selected snapshots of ion current behaviors of paced AP_i - AP_j collisions at CL = 1/60 min ($51.945 \leq \text{hERG blocker concentration} \leq 51.95$ nM) (Figure 8). Such collisions occur prior to the tipping point of spontaneous $\text{I}_{\text{Ca,L(late)}}$ recovery, which is restricted to longer CL (1/35 min in our study). Increasing perturbation severity results in AP_i - AP_j collisions at increasing AP_i $\Delta\psi_m(t)$ (the takeoff voltage of atypical AP_j depolarizations). The takeoff voltage ranges between -88 (normal resting) $\leq \Delta\psi_m(t) \leq -30$ mV. Atypical AP_j depolarizations are driven by

$I_{Ca,L}/I_{Na}$ mixtures (Figure 18A), which are counter-balanced largely by I_{K1} and I_{to} . The depolarization takeoff voltage increases with increasing perturbation severity, mirrored by increasing I_{Na} knockdown, I_{to} knockdown (~94% at blocker concentration ≈ 51.9504 nM and takeoff voltage = -75 mV (Figure 18B)), and I_{K1} knockdown (~50% at blocker concentration ≈ 51.9506 nM and takeoff voltage ≈ -60 mV; ~100% at blocker concentration ≈ 51.9510 nM and takeoff voltage ≈ -30 mV (Figure 18C)). $Ca_v1.2$ channels are distributed in the VDI and CDI states of AP_i prior to the collision, which recover in response to AP_j stimuli.

A

Figure 18. (A) $I_{Ca,L(peak)}$ increases from -4.8 to -9.7 (red tracing) pA/pF, whereas I_{Na} decreases from -237 to -6.3 pA/pF at hERG blocker concentration = 51.9504 nM (cyan tracing) versus control, respectively. (B) $I_{Ca,L}$, I_{K1} , and I_{to} profiles at hERG blocker concentration = 51.9504 nM (takeoff voltage = -75 mV). I_{K1} remains similar to control, whereas I_{to} is knocked down from ~ 3.1 to 0.2 pA/pF under control versus perturbed conditions, respectively. (C) $I_{Ca,L}$, I_{K1} , and I_{to} profiles at hERG blocker concentration = 51.95101 nM (takeoff voltage = -30 mV). I_{K1} is knocked down to ~ 0 , and I_{to} is knocked down from ~ 3.1 to 0.2 pA/pF under control versus perturbed conditions, respectively. $I_{Na(early)}$ is likewise knocked out, whereas the magnitude of $I_{Na(late)}$ is similar to control, but prolonged (not shown).

The effects of hERG dysfunction on ion currents in the APD/CL > 1 regime (hERG blocker concentration $\geq T_2$ @ CL = 1/60 min)

Next, we studied the ion current profiles at selected snapshots (Figure 8) of the paced AP_j-AP_k collision at $\geq T_2$ ($51.9511 \leq$ hERG blocker concentration ≤ 60.235 nM) (Figure 19). Similar results are expected at CL = 1/80 min. In this scenario, collisions result in Ca_v1.2 reopening, rather than recovery.

Figure 19. An atypical $I_{Ca,L(late)}$ -driven depolarization (AP_j) collides with the following paced AP (AP_k) at $CL = 1/60$ min and blocker concentration = 51.9565 nM ($k_{on} = 1e8 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{off} = 2.0 \text{ s}^{-1}$). This results in d gate reopening (light green tracing in the upper panel) in a small fraction of closed $Ca_v1.2$ channels (magenta arrow in the upper panel), which evokes an additional atypical $I_{Ca,L(late)}$ -driven depolarization (magenta arrow in the lower panel) conveyed largely by $I_{VCa,s}$ (bright green tracing in the upper panel). This differs from atypical AP_j depolarizations, which are driven by recovery of the CDI and VDI states from inactivation (noting that nca remains constant during AP_j - AP_k collisions).

hERG blockade dose-response relationships

We monitored the following mutually inclusive pro-arrhythmia contributions as a function of hERG blocker concentration:

- 1) Maximum peak fractional hERG blocker occupancy (or magnitude of other acquired or inherited pro-arrhythmic modalities).
- 2) Maximum APD/CL and variability thereof across a large AP series (representative of the degree of instability of the AP generation system), noting that APD alone is an indirect metric of pro-arrhythmic propensity (i.e. a passenger, rather than a driver).
- 3) Minimum $d(\Delta\psi_m(t))/dt$ centered at -30 mV (which mirrors the overall buildup of $I_{Ca,L}$ during AP phase 3) and variability thereof across a large AP series.
- 4) Maximum fold-increase in $|I_{VCa,f} + I_{V,f}|$ relative to control, and variability thereof across a large AP series.

We used ORd/hERG Markov simulations to characterize the aforementioned criteria for trappable and non-trappable hERG blockers in M cells as a function of concentration, BK, and CL (1/80, 1/60, 1/35 min). First, we used the automated algorithm described in Materials and methods to determine the PT_1 threshold in each scenario, which serves as the endpoint in our dose-response simulations. The results, presented in Table 1 and Figures 20-23, may be summarized as follows:

- 1) Sensitivity of the AP generation system to perturbation grows with CL in order of $1/80 > 1/35 > 1/60$ min, suggesting the existence of an anti-arrhythmic Goldilocks zone of CL centered at 1/60 min.
- 2) A “shockwave” of instability builds within the first ~100 APs at blocker concentration = PT_1 , settling to a new metastable state after ~450 APs (Figure 20G), suggesting that perturbation levels $< T_1$ are buffered by the AP generation system.

- 3) Variability of APD/CL, $d(\Delta\psi_m(t))/dt$, and $|I_{Vca,f} + I_{V,f}|$ grows as a function of time, which is mirrored by short-long-short sequences in the ECG preceding the onset of organ level arrhythmia.

A

B

C

D

E

F

G

Figure 20. (A) Upper panel: APD/CL at CL = 1/35 min for control (blue tracing) versus non-trappable hERG blocker $k_{on} = 2.0e6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 2.0 \text{ s}^{-1}$, blocker concentration = $PT_1 = 2,218.66818 \text{ nM}$ rounded to five decimal places (blue tracing). Oscillations in APD/CL build throughout the latter half of the simulation (red arrow), reflecting increasing instability of the system, which nevertheless remains non-arrhythmic at this perturbation level. Lower panel: Distribution of APD/CL across the 200 APs of the simulation under control versus perturbed conditions (blue and red bars, respectively). The broad distribution of APD/CL under perturbed conditions reflects high instability of the system in the PT_1 regime. (B) Same as A, except CL = 1/60 min and $PT_1 = 2,597.4567 \text{ nM}$. (C) Same as A, except CL = 1/80 min and $PT_1 = 2,448.6258 \text{ nM}$. (D) Same as A, except $k_{on} = 1.0e8 \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 2.0 \text{ s}^{-1}$, and $PT_1 = 1,203.3485 \text{ nM}$. (E) Same as D, except CL = 1/60 min and $PT_1 = 1,398.5543 \text{ nM}$. (F) Same as D, except CL = 1/80 min and $PT_1 = 1,259.2552 \text{ nM}$. (G) Same as C, except $PT_1 = 2,448.1306 \text{ nM}$ and the number of simulated APs = 1,000. Overt instability present in the previous tracings (resembling a shockwave) settles to

a new metastable non-arrhythmic steady state after ~450 APs. We further note that PT_1 is significantly higher at 60 versus 35 and 80 BPM (Table 1).

A

B

C

D

Figure 21. (A) Upper panel: Same as Figure 20A, except showing control versus perturbed $d(\Delta\psi_m(t))/dt$ (blue and red tracings, respectively). Fold-difference in perturbed/control is shown in magenta. Lower panel: Same as Figure 20A, except showing control versus perturbed

$d(\Delta\psi_m(t))/dt$. (B) Same as A, except $CL = 1/60$ min and $PT_1 = 2,597.4567$ nM. (C) Same as A, except $CL = 1/80$ min and $PT_1 = 2,448.6258$ nM. (D) Same as C, except showing the last 16/200 APs of the simulation under control (blue) versus perturbed (red) conditions, with $d(\Delta\psi_m(t))/dt$ tangent lines (red vectors) superimposed on the perturbed APs (slope calculation method described in Materials and methods). The AP numbers in the upper panel (corresponding to those on the x-axis of the lower panel) are shown in bold.

A

B

C

Figure 22. (A) Upper panel: Same as Figure 20A, except showing control versus perturbed $|I_{VCa,f}|$, $|I_{V,f}|$, $|I_{VCa,f} + I_{V,f}|$, and $(|I_{VCa,f}| + |I_{V,f}|)$ perturbed/control (color coding noted in the legend).

Lower panel: Same as Figure 20A, except showing $|I_{VCa,f} + I_{V,f}|$. (B) Same as A, except CL =

1/60 min and $PT_1 = 2,597.4567$ nM. (C) Same as A, except $CL = 1/80$ min and $PT_1 = 2,448.6258$ nM.

A

B

Figure 23. (A) I_{Kr} , $I_{Vca,f}$, $I_{V,f}$, and fractional blocked and free hERG populations at the 200th AP (mapped to the far right position in the upper panel of Figure 21D). Inset: zoomed in view of the current tracings, showing the rates of buildup of the open + inactivated channel population (blue arrow) and blocker occupancy (red arrow). Peak occupancy is also shown (red dotted line). CL = 1/35 min and blocker $k_{on} = 2.0e6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 2.0 \text{ s}^{-1}$, blocker concentration = $PT_1 = 2,218.66818 \text{ nM}$ (color coding noted in the legend). Late $I_{Vca,f}$ (solid red tracing in the negative direction) is far above control, I_{Kr} is approximately 50% of control (equal to the unblocked hERG fraction). Buildup of the blocker-occupied channel population (solid red tracing in the positive direction) lags behind channel activation/inactivation at this comparatively slow k_{on} , and decay of the occupied population lags behind channel deactivation (solid blue curve), peaking at ~50% of the total population. We hypothesize that arrhythmia is driven by peak, rather than fractional AUC occupancy (AUC of the bound population/AUC of the open + inactivated population $\approx 43\%$ in this scenario). (B) Same as A, except $k_{on} = 2.0e6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{off} = 2.0 \text{ s}^{-1}$, and blocker concentration = $PT_1 = 1,203.3490 \text{ nM}$. The lag in buildup of the bound state is considerably reduced by faster k_{on} compared with that in A, and consequently, the peak occupancy lifetime is longer (recalling that $IC_{50} = 1.0 \text{ }\mu\text{M}$ in both scenarios). These observations support our claim that occupancy is kinetically, rather than thermodynamically, driven.

Next, we simulated APs for each BK and CL scenario over a series of blocker concentrations ranging from 0 to PT_1 in 100 nM increments (finer increments were sampled proximal to PT_1 due to the highly non-linear behavior of the pro-arrhythmic criteria within this regime). The results, which are plotted in Figures 24-25, may be summarized as follows:

- 1) Maximum hERG occupancy as a function of blocker concentration across each simulation builds to the 50-60% level at PT_1 according to the Hill equation for all trappable and non-trappable blockers exhibiting $k_{on} \geq 1.0e8 \text{ M}^{-1} \text{ s}^{-1}$. However, occupancy builds more slowly than the rate of channel opening and decays faster than the rate of channel closing for non-trappable blockers exhibiting $k_{on} \leq 1.0e7 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 23A versus Figure 23B), as explained in our previous work [12,19] (reflected in distinct banding in Figure 24A). hERG occupancy drives all pro-arrhythmic effects by tipping the dynamic net inward-outward current balance toward the inward direction (in a graded fashion up to slightly below PT_1), which in turn, slows Kir2.1 activation, and thereby generates an anomalous $I_{Ca,L(late)}$ window current. At longer CL, $I_{Ca,L(late)}$ builds to the spontaneous depolarizing level, reaching this level at shorter CL via APi-APj collisions (paced stimulation). Further increases in blocker concentration $> T_3$ result in sustained cellular arrhythmia.
- 2) Maximum APD/CL (Figure 20) grows quasi-linearly across each simulation as a function of blocker occupancy, becoming highly non-linear within a small increment below PT_1 (Figure 24B). At CL = 1/60 and 1/80 min, APD/CL approaches 1.0 at PT_1 , whereas at CL = 1/35 min, APD/CL approaches ~0.5 at PT_1 , reflecting the differing pro-arrhythmic mechanisms at longer versus shorter CL (Figure 5A). Furthermore, APD/CL grows more slowly with increasing blocker concentration for non-trappable blockers exhibiting $k_{on} \leq 1.0e7 \text{ M}^{-1} \text{ s}^{-1}$ compared with all trappable blockers and non-trappable blockers exhibiting faster k_{on} (e.g. a non-trappable blocker exhibiting $k_{on} = 2.0e6 \text{ M}^{-1} \text{ s}^{-1}$ reaches PT_1 at approximately twice the concentration of a trappable blocker exhibiting the same k_{on} at CL = 1/35 min).

- 3) A steeper quasi-linear relationship exists between blocker concentration and minimum $d(\Delta\psi_m(t))/dt$ across each simulation (Figure 24C), while retaining the biphasic morphology of the other dose-response tracings. At a given blocker concentration, $d(\Delta\psi_m(t))/dt$ decreases throughout each simulation (Figure 21D), the overall minimum of which approaches zero at PT_1 . APD and Kir2.1 activation are governed directly, and $I_{Ca,L(late)}$ directly by $d(\Delta\psi_m(t))/dt$.
- 4) $(\text{total } |I_{Vca,f} + I_{V,f}| \text{ perturbed AUC})/(\text{total } |I_{Vca,f} + I_{V,f}| \text{ control AUC})$, equating to the fold increase in $I_{Ca,L(late)}$ above control (calculated within a time window between AP start + 150 ms and AP end) serves as a direct window into the act of cellular arrhythmogenesis (Figure 24D). $I_{Ca,L(late)}$ at PT_1 grows by ~2-fold at $CL = 1/60$ and $1/80$ min and ~5-fold at $CL = 1/35$ min.
- 5) Biphasic behavior of hERG blockade dose-response curves is analogous to pH buffering in acid-base titration, in which large perturbations are absorbed until near saturation limit of the system (exemplified in Figure 24E). However, unlike acid-base titration, pro-arrhythmic behaviors undergo high frequency oscillations on approach to PT_1 (exemplified in Figure 20G), reflecting high instability of the AP generation system.
- 6) The relationships between the key arrhythmia-conveying parameters $d(\Delta\psi_m(t))/dt$ versus $(\text{total } |I_{Vca,f} + I_{V,f}| \text{ perturbed AUC})/(\text{total } |I_{Vca,f} + I_{V,f}| \text{ control AUC})$ and hERG occupancy versus $(\text{total } |I_{Vca,f} + I_{V,f}| \text{ perturbed AUC})/(\text{total } |I_{Vca,f} + I_{V,f}| \text{ control AUC})$ are shown in Figures 25A and B, respectively.

7) Snapshots of key ion current and $\text{Ca}_v1.2$ gating effects at arbitrary BK profile and sub-PT₁ hERG blocker concentrations (500 and 1,000 nM) are shown in Figures 26A and B, respectively.

A

B

C

D

E

Figure 24. Dose-response tracings for the pro-arrhythmia criteria described in the text. The final concentration in each plot corresponds to PT_1 . (A) Dose-peak hERG occupancy relationships for each of the BK and CL scenarios listed above (color-coding as denoted in the legend). (B) Same as A, except showing dose-APD/CL relationships. (C) Same as A, except showing dose- $d(\Delta\psi_m(t))/dt$ relationships. (D) Same as A, except showing dose-(total $|I_{V_{Ca,f}} + I_{V,f}|$ perturbed)/(total $|I_{V_{Ca,f}} + I_{V,f}|$ control). (E) The dose-response tracings in B, C, and D resemble generic acid-base titration in the presence of a buffer (Lasse Havelund, <https://creativecommons.org/licenses/by-sa/4.0/deed.en>).

A

B

Figure 25. (A) Perturbed minimum $d(\Delta\psi_m(t))/dt$ plotted against (total $|I_{Vca,f} + I_{V,f}|$ perturbed)/(total $|I_{Vca,f} + I_{V,f}|$ control) (color-coding noted in the legend). A quasi-hyperbolic relationship between these quantities is apparent (consistent with the asymptotic approach of $d(\Delta\psi_m(t))/dt$ to 0). The different CL-dependent mechanisms of atypical depolarization result in the appearance of two distinct bands in the plot. (B) Peak fractional hERG occupancy plotted against (total $|I_{Vca,f} + I_{V,f}|$ perturbed)/(total $|I_{Vca,f} + I_{V,f}|$ control). A quasi-hyperbolic relationship between these quantities suggests that hERG occupancy approaches a maximum at the T_1 threshold of $Ca_v1.2$ recovery.

A

B

Figure 26. (A) Upper left panel: snapshot of the $I_{V_{Ca,f}}$ window current and $f_{Ca,f}$ gate under control conditions (dotted tracings) and in the presence of a non-trappable hERG blocker exhibiting $k_{on} = 2.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{off} = 2.0 \text{ s}^{-1}$ at a concentration = 500 nM (solid tracings). Lower left panel: prolonged and control AP (blue and orange tracings, respectively) corresponding to the tracings in the upper panel. Upper right panel: hERG occupancy (red tracing) I_{K_r} (light blue tracing) and I_{K_s} (green tracing) under control (dotted tracings) and perturbed conditions (solid tracings). Lower right panel: same as lower left panel. (B) Upper left panel: snapshot of all contributions to the $I_{C_{a,L(late)}}$ window current under control conditions (dotted tracings) and in the presence of the hERG blocker described in A (solid tracings), except at 1,000 nM. $I_{V_{Ca,f}}$ differs the most from control among these currents, accounting for the bump in the $I_{C_{a,L(late)}}$ tracing that grows to the depolarizing level at the T_1 threshold of blockade. Lower left panel: same as lower left pane in A. Upper right panel: $I_{C_{a,L(late)}}$ gating under control (dotted tracings) and perturbed conditions (solid tracings) underlying the anomalous window current. Lower right panel: same as upper right panel in A.

Table 1. Magnitudes of the key pro-arrhythmia criteria at hERG blocker concentration = PT_1 (determined iteratively via the algorithm described in Materials and methods) as a function of k_{on} , k_{off} , and CL at a fixed $IC_{50} = 1.0 \mu M$ (calculated parameters at $k_{off} < k_{off_trappable_floor}$ and $k_{off_non-trappable_floor}$ have been omitted). Scalar quantities were calculated from the maxima of each parameter over the 200 APs of the simulations for control (denoted as C) and perturbed (denoted as (P) conditions. The quantities include: 1) maximum peak blocker occupancy (denoted as γ_{T_1}) calculated using ORd/hERG Markov simulations and equilibrium occupancy (denoted as γ_{eq}) calculated using the Hill equation (noting that $\gamma_{T_1} = \gamma_{eq}$ for trappable blockers, and γ_{T_1} converges to γ_{eq} for non-trappable blockers exhibiting $k_{on} \gtrsim 1e8 M^{-1} s^{-1}$); 2) PT_1 (rounded to five decimal places); 3) minimum $d(\Delta\psi_m(t))/dt$ during AP phase 3; 4) spread in AP slope represented by the number of histogram bins in the dataset; 5) maximum $|I_{Ca,L}|$; 6) fold-increase in maximum total late $|I_{VCa,f} + I_{V,f}|$ over control (represented by the area under the current over time tracing); 7) spread in the maximum total late $|I_{VCa,f} + I_{V,f}|$; 8) maximum APD and APD/CL ; 9) spread in the maximum APD.

		1/35 (min)	1/60 (min)	1/80 (min)	k_{on} (M⁻¹ s⁻¹)	k_{off} (s⁻¹)
T	Y_{T1}, Y_{eq} (%)	53.94, 53.94	60.11, 60.11	55.28, 55.28	6.93e5	0.693*
	PT₁ (nM)	1,171.24906	1,506.7279	1,236.3398		
	Min $d(\Delta\psi_m(t))/dt$ (mV/ms)	-0.035 (P) -0.499 (C)	-0.047 (P) -0.533 (C)	-0.077 (P) -0.534 (C)		
	Distribution	25 (P) 11 (C)	25 (P) 6 (C)	34 (P) 9 (C)		
	Max I_{Ca,L} (pA/pF)	4.79 (P) 4.78 (C)	4.72 (P) 4.73 (C)	5.98 (P) 5.0 (C)		
	Max AUC total $I_{Vca,f} + I_{V,f}$ (P/C)	6.66	2.79	1.95		
	Distribution	39	19	10		
	Max APD (ms)	920.83 (P) 396.13 (C)	814.24 (P) 363.29 (C)	708.15 (P) 361.39 (C)		
	Distribution	44 (P) 7 (C)	42 (P) 1 (C)	37 (P) 4 (C)		
	Max APD/CL	0.537 (P) 0.231 (C)	0.814 (P) 0.363 (C)	0.944 (P) 0.482 (C)		

NT	Y_{T1}, Y_{eq} (%)	50.31, 68.93	53.13, 72.20	51.26, 71.0	2.0e6	2.0**
	PT₁ (nM)	2,218.66818	2,597.4567	2,448.6258		
	Min $d(\Delta\psi_m(t))/dt$ (mV/ms)	-0.089 (P) -0.499 (C)	-0.021 (P) - 0.533 (C)	-0.067 (P) -0.534 (C)		
	Distribution	16 (P) 11 (C)	14 (P) 6 (C)	29 (P) 9 (C)		
	Max I_{Ca,L} (pA/pF)	4.77 (P) 4.79 (C)	6.0 (P) 4.73 (C)	5.51 (P) 5.0 (C)		
	Max AUC total $I_{VCa,f} + I_{V,f}$ (P/C)	5.29	1.99	2.07		
	Distribution	35	6	9		
	Max APD (ms)	762.13 (P) 396.13 (C)	964.57 (P) 363.29 (C)	689.17 (P) 361.39 (C)		
	Distribution	34 (P) 7 (C)	13 (P) 1 (C)	34 (P) 4 (C)		
	Max APD/CL	0.445 (P) 0.231 (C)	0.965 (P) 0.363 (C)	0.919 (P) 0.482 (C)		

T	$\gamma_{T_1}, \gamma_{eq}$ (%)	53.96, 53.96	59.92, 59.92	55.27, 55.27	2.0e6	2.0**
	PT_1 (nM)	1,171.80766	1,494.9204	1,235.7236		
	Min $d(\Delta\psi_m(t))/dt$ (mV/ms)	-0.060 (P) -0.499 (C)	-0.042 (P) -0.533 (C)	-0.079 (P) -0.533 (C)		
	Distribution	21 (P) 11 (C)	22 (P) 6 (C)	30 (P) 9 (C)		
	Max $I_{Ca,L}$ (pA/pF)	4.77 (P) 4.79 (C)	4.94 (P) 4.73 (C)	5.98 (P) 5.00 (C)		
	Max AUC total $I_{Vca,f} + I_{V,f}$ (P/C)	4.88	2.61	1.94		
	Distribution	33	15	8		
	Max APD (ms)	821.28 (P) 396.13 (C)	901.7 (P) 363.29 (C)	706.48 (P) 361.39 (C)		
	Distribution	38 (P) 7 (C)	37 (P) 1 (C)	33 (P) 4 (C)		
	Max APD/CL	0.479 (P) 0.231 (C)	0.902 (P) 0.363 (C)	0.942 (P) 0.482 (C)		

NT	γ_1, γ_{eq} (%)	57.1, 59.39	59.67, 62.13	56.47, 59.15	1.0e7	1.0e1
	PT₁ (nM)	1,462.17	1,640.3048	1,447.9159		
	Min $d(\Delta\psi_m(t))/dt$ (mV/ms)	-0.034 (P) -0.499 (C)	-0.03 (P) -0.534 (C)	-0.068 (P) -0.534 (C)		
	Distribution	20 (P) 11 (C)	15 (P) 6 (C)	30 (P) 9 (C)		
	Max I_{Ca,L} (pA/pF)	4.77 (P) 4.79 (C)	6.0 (P) 4.73 (C)	5.98 (P) 5.00 (C)		
	Max AUC total $I_{Vca,f} + I_{V,f}$ (P/C)	5.43	1.98	2.05		
	Distribution	35	7	9		
	Max APD (ms)	873.87 (P) 396.13 (C)	964.86 (P) 363.29 (C)	705.92 (P) 361.39 (C)		
	Distribution	37 (P) 7 (C)	15 (P) 1 (C)	34 (P) 4 (C)		
	Max APD/CL	0.051 (P) 0.231 (C)	0.965 (P) 0.363 (C)	0.941 (P) 0.482 (C)		

T	$\gamma_{T_1}, \gamma_{eq}$ (%)	53.92, 53.92	57.7, 57.7	55.27, 55.27	1.0e7	1.0e1
	PT_1	1,170.22907	1,364.095	1,235.5668		
	Min $d(\Delta\psi_m(t))/dt$ (mV/ms)	-0.074 -0.499	-0.04 (P) -0.533 (C)	-0.079 (P) -0.534 (C)		
	Distribution	18 (P) 11 (C)	14 (P) 6 (C)	27 (P) 9 (C)		
	Max $I_{Ca,L}$ (pA/pF)	4.77 (P) 4.79 (C)	6.0 (P) 4.73 (P)	5.92 (P) 5.0 (C)		
	Max AUC total $I_{Vca,f} + I_{V,f}$ (P/C)	4.65	1.86	1.94		
	Distribution	31	6	8		
	Max APD (ms)	778.19 (P) 396.13 (C)	964.14 (P) 363.29 (C)	704.8 (P) 361.39 (C)		
	Distribution	37 (P) 7 (C)	13 (P) 1 (C)	34 (P) 4 (C)		
	Max APD/CL	0.454 (P) 0.231 (C)	0.964 (P) 0.363 (C)	0.94 (P) 0.482 (C)		

NT	$\gamma_{T_1}, \gamma_{eq}$ (%)	54.49, 54.62	58.18, 58.31	55.61, 55.74	1.0e8	1.0e2
	PT₁ (nM)	1,203.3485	1,398.5543	1,259.2552		
	Min $d(\Delta\psi_m(t))/dt$ (mV/ms)	-0.077 (P) -0.499 (C)	-0.039 (P) -0.533 (C)	-0.076 (P) -0.534 (C)		
	Distribution	18 (P) 11 (C)	14 (P) 6 (C)	29 (P) 9 (C)		
	Max I_{Ca,L} (pA/pF)	4.79 (P) 4.77 (C)	6.0 (P) 4.73 (C)	5.81 (P) 5.0 (C)		
	Max AUC total $I_{Vca,f} + I_{V,f}$ (P/C)	4.66	1.89	1.95		
	Distribution	31	6	8		
	Max APD (ms)	784.1 (P) 396.13 (C)	964.40 (P) 363.29 (C)	701.71 (P) 361.39 (C)		
	Distribution	35 (P) 7 (C)	14 (P) 1 (C)	35 (P) 4 (C)		
	Max APD/CL	0.457 (P) 0.231 (C)	0.964 (P) 0.363 (C)	0.936 (P) 0.482 (C)		

T	γ_{T1}, γ_{eq} (%)	53.96, 53.96	57.77, 57.77	55.26, 55.26	1.0e8	1.0e2
	PT₁ (nM)	1,171.8390	1,367.7385	1,235.301		
	Min $d(\Delta\psi_m(t))/dt$ (mV/ms)	-0.033 (P)	-0.04 (P)	-0.078 (P)		
		-0.499 (C)	-0.533 (C)	-0.534 (C)		
	Distribution	21 (P)	13 (P)	29 (P)		
		11 (C)	6 (C)	9 (C)		
	Max I_{Ca,L} (pA/pF)	4.77 (P)	6.0 (P)	5.95 (P)		
		4.79 (C)	4.73 (C)	5.0 (C)		
	Max AUC total $I_{Vca,f} + I_{V,f}$ (P/C)	5.40	1.86	1.94		
	Distribution	35	6	8		
	Max APD (ms)	923.15 (P)	964.74 (P)	706.27 (P)		
		396.13 (C)	363.29 (C)	361.39 (C)		
	Distribution	40 (P)	13 (P)	33 (P)		
		7 (C)	1 (C)	4 (C)		
	Max APD/CL	0.539 (P)	0.965 (P)	0.942 (P)		
		0.231 (C)	0.363 (C)	0.482 (C)		

NT	$\gamma_{T_1}, \gamma_{eq}$ (%)	53.92, 54.0	57.73, 57.81	55.23, 55.31	1.0e9	1.0e3
	PT₁ (nM)	1,173.73975	1,370.41727	1,237.5011		
	Min $d(\Delta\psi_m(t))/dt$ (mV/ms)	-0.066 (P) -0.499 (C)	-0.037 (P) -0.535 (C)	-0.078 (P) -0.534 (C)		
	Distribution	19 (P) 11 (C)	13 (P) 6 (C)	28 (P) 9 (C)		
	Max I_{Ca,L} (pA/pF)	4.77 (P) 4.79 (C)	6.0 (P) 4.73 (C)	5.95 (P) 5.0 (C)		
	Max AUC total $I_{Vca,f} + I_{V,f}$ (P/C)	4.78	1.87	1.95		
	Distribution	32	6	8		
	Max APD (ms)	802.65 (P) 396.13 (C)	964.72 (P) 363.29 (C)	705.55 (P) 361.39 (C)		
	Distribution	37 (P) 7 (C)	14 (P) 1 (C)	35 (P) 4 (C)		
	Max APD/CL	0.468 (P) 0.231 (C)	0.965 (P) 0.363 (C)	0.941 (P) 0.482 (C)		

T	$\gamma_{T_1}, \gamma_{eq}$ (%)	53.93, 53.93	57.77, 57.77	55.26, 55.26	1.0e9	1.0e3
	PT_1 (nM)	1,170.52515	1,368.07097	1,235.244		
	Min $d(\Delta\psi_m(t))/dt$ (mV/ms)	-0.073 (P) -0.507 (C)	-0.04 (P) -0.535 (C)	-0.079 (P) -0.534 (C)		
	Distribution	18 (P) 11 (C)	14 (P) 6 (C)	29 (P) 9 (C)		
	Max $I_{Ca,L}$ (pA/pF)	4.69 (P) 4.79 (C)	6.0 (P) 4.73 (C)	5.97 (P) 5.00 (C)		
	Max AUC total $I_{Vca,f} + I_{V,f}$ (P/C)	4.59	1.86	1.95		
	Distribution	31	6	8		
	Max APD (ms)	795.06 (P) 396.13 (C)	964.40 (P) 363.29 (C)	707.24 (P) 361.39 (C)		
	Distribution	37 (P) 7 (C)	13 (P) 1 (C)	35 (P) 4 (C)		
	APD/CL	0.464 (P) 0.231 (C)	0.964 (P) 0.363 (C)	0.943 (P) 0.482 (C)		

* $k_{off_trappable_floor}$; ** $k_{off_non-trappable_floor}$.

As expected, the T_1 threshold differs between $APD/CL < 1$ (corresponding to the spontaneous depolarizing threshold of the $I_{Ca,L}$ window current), and $APD/CL \rightarrow 1$ (corresponding to recovery of $Ca_v1.2$ channels from the CDI and VDI states triggered by paced AP_i - AP_j collisions). The results of our simulations suggest that the effects of blockade on $I_{Ca,L}$, including the CDI state population level (nca), f_{Ca} gating, $I_{V_{Ca,f}}$ window current, APD/CL , and shoulder $d(\Delta\psi_m(t))/dt$ (a putative “pro-arrhythmia fingerprint”) may serve as a more direct metric of pro-arrhythmic propensity than both APD and QT prolongation.

Na_v1.5 loss of inactivation function (LOF)-induced arrhythmogenesis in LQT3

Next, we set about to understand the pro-arrhythmic and arrhythmic effects of $I_{Na(late)}$ gain of function (Figure 27), which we simulated by slowing the rate of channel inactivation, as described in Materials and methods (equations 4 and 5). We manually titrated $h_{L,ss}$ to the PT_1 threshold via adjustment of V_{shift} at $CL = 1/35, 1/60$, and $1/80$ min (Table 2). The lowest V_{shift} at this threshold is achieved at $CL = 1/35$ min (i.e. bradycardic conditions), consistent with the known trigger of arrhythmia in LQT3 (which is exacerbated by the sinoatrial node suppressing effects of LQT3 mutations [20]). This may be explained by the presence of intact repolarizing currents in this scenario (compared with hERG blockade and LQT2), which affords greater capacity for I_{K1} activation during AP-AP collisions at shorter CL. A snapshot of $I_{V_{Ca,f}}$ at a single prolonged AP at PT_1 is shown in Figure 28A, and APD/CL and $(\text{total } |I_{V_{Ca,f}} + I_{V,f}| \text{ perturbed AUC})/(\text{total } |I_{V_{Ca,f}} + I_{V,f}| \text{ control AUC})$ are plotted over the 200 APs of the simulation in Figure 28B.

Figure 27. $V_{shift} = -74.1893$ at CL = 1/35 min. (A) $I_{Na(ate)}$ under perturbed and control conditions (blue and orange tracings, respectively). The maximum perturbed $I_{Na(ate)}$ during AP phase 3 is approximately 3-fold greater than control (~ 3.2 versus -0.1 pA/pF).

Table 2. V_{shift} titration to the PT₁ level (performed manually, unlike in our hERG blocker dose-response studies).

V_{shift} @ PT ₁ (mV)	CL (min)
-74.1893	1/35
-79.4772	1/60
-80.4458	1/80

A

B

Figure 28. $V_{shift} = -74.1893$ at CL = 1/35 min. (A) Upper panel: A representative highly prolonged AP (red) versus control (red and blue, respectively), together with their slopes centered at -30 mV. Lower panel: Repolarizing currents and $I_{VCa,f}$ (color-coding denoted in the legend). $I_{VCa,f}$ is significantly larger and broader than control at this V_{shift} .

hERG gain of inactivation function (GOF)-induced arrhythmogenesis in LQT2

Next, we set about to understand the mechanism of I_{Kr} knockdown-driven arrhythmia, which we simulated by separately speeding the rate of hERG inactivation (α_i) and decreasing the rate of recovery (β_i) in the Markov model, as described in Materials and methods (equations 1 and 2, respectively). We manually titrated α_i and β_i to the PT_1 threshold via adjustment of $\alpha_i V_{shift}$ and $\beta_i V_{shift}$ (Tables 3 and 4, respectively). The resulting effects on I_{Kr} are shown in Figure 29.

Figure 29. Upper panel: Time-dependent hERG inactivation, I_{Kr} , and I_{K1} under control and $\alpha_i V_{shift} = PT_1 = 65.0538$ mV (light blue and green, orange and blue, and purple tracings, respectively) conditions at $CL = 1/35$ min. Lower panel: $(\Delta\psi_m(t))$ under control and perturbed conditions (orange and blue tracings, respectively). Nearly identical behavior occurs at $\beta_i V_{shift} = 106.178925$ mV.

Table 3. $\alpha_i V_{shift}$ titration to the PT_1 level.

$\alpha_i V_{shift}$ @ PT_1 (mV)	CL (min)
65.0538	1/35
66.8892	1/60
63.0618	1/80

Table 4. $\beta_i V_{shift}$ titration to the PT_1 level.

$\beta_i V_{shift}$ @ PT_1 (mV)	CL (min)
106.17925	1/35
109.26436	1/60
102.93924	1/80

As for hERG blockade, the PT_1 level for both sped inactivation and slowed recovery is greatest at $CL = 1/60$ min, which further supports the possibility of enhanced arrhythmia resistance at this CL. Furthermore, far greater sensitivity to sped inactivation versus slowed recovery is apparent (noting that these effects are non-mutually exclusive and may occur in combination in LQT2-afflicted individuals). The current effects at PT_1 are shown in Figure 30.

A

B

Figure 30. (A) Upper panel: Simulated APs 2-8 under control (blue) versus $\alpha_{i-V_{shift}} = PT_1 = 65.0538$ mV (red) conditions at CL = 1/35 min, with $d(\Delta\psi_m(t))/dt$ tangent lines (red vectors) superimposed on the perturbed APs (slope calculation method described in Materials and methods). The AP numbers in the upper panel (corresponding to those on the x-axis of the lower panel) are shown in bold. Lower panel: Plot of $d(\Delta\psi_m(t))/dt$ centered at -30 mV as a function of time. (B) Upper panel: APD/CL as a function of time under control versus perturbed conditions (blue and red tracings, respectively). Lower panel: $|I_{V_{Ca,f}}|$, $|I_{V,f}|$, $|I_{V_{Ca,f}} + I_{V,f}|$, and $(|I_{V_{Ca,f}}| + |I_{V,f}|)$ perturbed/control (color coding noted in the legend).

Arrhythmogenesis is graded according to hERG blockade, LQT2, and LQT3 severity

Lastly, we examined the progression of arrhythmia at perturbation levels $\geq T_1$ (versus PT_1) as a function of time and CL = 1/35 and 1/60 min. As described at length in the previous sections, arrhythmogenesis is fueled by the anomalous buildup of $I_{Ca,L}$ (principally $I_{V_{Ca,f}} + I_{V,f}$) to depolarizing levels as a function of time and perturbation severity, and conveyed by paced AP-AP collisions at CL = 1/35 min and spontaneous depolarizations at CL = 1/60 and 1/80 min. Both forms of pro-arrhythmia similarly progress to full arrhythmia in a graded fashion as a function of time and perturbation severity. Chronic, sub-acute pro-arrhythmic perturbations necessarily reside in the quasi-linear sub- PT_1 regime, escalating to the T_1 level and beyond in a condition-dependent manner, either due to downward shifting of the thresholds or increased perturbation severity in the case of hERG blockade (consistent with the stochastic nature of arrhythmia). Examples include changes in CL, hERG blocker exposure due to drug-drug interactions (DDIs)/overdose, physical activity, adrenergic stimulation, CaMKII activity, etc.

Our results suggest that arrhythmogenesis begins with graded instability of the AP generation system. Whereas a background of sub-arrhythmic instability exists below the PT_1 threshold, further escalation of the perturbation level to PT_1 results in a shockwave (Figure 20G), in which APD/CL , $d(\Delta\psi_m(t))/dt$, and $I_{Vca,f} + I_{V,f}$ undergo high frequency non-arrhythmic oscillations that: 1) settle to a new metastable state at PT_1 ; 2) progress to sporadic arrhythmic waveforms at T_1 and T_2 (at shorter CL); and 3) progress to lengthy or sustained arrhythmic wave trains (consisting of tetrads of atypical $I_{Ca,L}$ -driven depolarization cycles at $CL = 1/35$ min) at T_3 (Figure 31). This process is exemplified for simulated LQT3 (noting that hERG blockade and loss of hERG inactivation function behave similarly at this CL), as follows:

- 1) Paced + non-paced AP dyads occur sporadically at perturbation levels $\geq T_1$ and $CL = 1/35$ min (Figure 32A).
- 2) AP triads and tetrads resulting from additional atypical $I_{Ca,L}$ -driven depolarizations (reflecting oscillations in $Ca_v1.2$ inactivation gating shown in Figure 14) appear over time (Figures 32B and 32C).
- 3) The T_2 threshold is crossed when the wavelength of arrhythmic wave packets expands to the CL (corresponding to total loss of restitution time) (Figure 32C), at which point collisions with succeeding paced APs occur (Figure 32D).
- 4) The wave packet wavelength further expands at the T_3 threshold to $\gg CL$ (Figure 33), becoming continuous at perturbation levels $\gg T_3$ (Figure 34).

Figure 31. $V_{shift} = -77.0$ (simulated LQT3) at CL = 1/35 min. (A) Upper panel: Arrhythmic wave packets that are terminated by intact I_{Kr} , I_{Ks} , and I_{K1} activation at this perturbation level. Lower panel: “Daisy-chained” repolarizing currents above the T_1 threshold, which consist principally of $I_{Vca,f}$, and $I_{V,f}$ (color-coding denoted in the legend).

A

B

C

D

Figure 32. LQT3 $V_{shift} = -77.0$ mV (2.81 mV above PT_1) at $CL = 1/35$ min. Upper panels: $I_{Na(ate)}$ under perturbed and control conditions (blue and orange tracings, respectively). Lower panels: $\Delta\psi_m(t)$ under perturbed and control conditions (blue and orange tracings, respectively). At this perturbation level, the intra-packet frequency of spontaneous depolarizations increases over time from singlets (B) to doublets (C), triplets (D), and quartets (E) sandwiched between pairs of consecutive paced APs. Quartets are formed by out-of-phase collisions, resulting in bifurcations of intra-packet waveforms, as noted in the inset.

Figure 33. Same as Figure 29, except $V_{shift} = -79.0$ mV (4.81 mV above PT_1) at $CL = 1/35$ min.

The intra-wave packet wavelength and frequency expand across three paced APs at this perturbation level, resulting in additional collisions and bifurcations.

Figure 34. Same as Figure 29, except $V_{shift} = -83.0$ mV (8.81 mV above PT_1) at $CL = 1/35$ min.

The intra-wave packet wavelength and frequency expand continuously across the paced APs at this perturbation level.

Atypical spontaneous $I_{Ca,L}$ -driven depolarizations give way to pacing-triggered depolarizations at $CL = 1/60$ and $1/80$ min. The T_1 and T_2 stages of this process are shown for hERG blockade in Figures 8 and 35.

A

B

Figure 35. (A) The time-dependent progression of arrhythmic waveforms at $CL = 1/60$ min and hERG blocker concentration = 60.235 nM ($k_{on} = 1e8$, $k_{off} = 2.0$) (AP 1) to single pacing-triggered atypical $I_{Ca,L}$ -driven depolarizations (wave packets 2-5) to collision-induced AP dyads (wave

packet 7). (B) Same as A, except hERG blocker concentration = 65.0 nM. The APs progress from a collision-induced bifurcation (wave packet 1), which evokes additional pacing-triggered atypical depolarizations (wave packets 2 and 3). This process further expands (wave packet 4), becoming continuous at wave packet 6.

Discussion

The implications of our findings for electrical homeostasis

We recently described a first principles physics-based theory (which we refer to as “Biodynamics” [1,21]) describing the general paradigm by which:

- 1) Cellular function (biochemical and electrical) is generated by molecular systems.
- 2) Molecular systems respond to perturbations.
- 3) Cellular dysfunction results from molecular dysfunction or excessive perturbation.

Here, we have used Biodynamics to explain arrhythmogenesis in, as well as the natural anti-arrhythmic/homeostatic properties of, ventricular cardiomyocytes based on perturbation-induced alterations in the dynamic net inward-outward current balance as a function of CL. Our results suggest that cellular homeostasis/arrhythmia resistance depends first and foremost on a Goldilocks zone of APD/CL across all CL (0.23, 0.36, and 0.48 under control conditions in our simulations at pacing CL = 35, 60, and 80 BPM, respectively). APDs shorten and lengthen with decreased and increased pacing CL, respectively. APD is governed by processes operating at both the intra- and extra-cycle levels (the latter of which are neglected in the ORd model). Intra-cycle processes include CaMKII-mediated phosphorylation and phosphatase-mediated dephosphorylation of ion channels and RyR₂ (which speed and slow inactivation, respectively), whereas extra-cycle

processes (acting on timescales $\gg$ a single CL) include β 2-mediated PIP₂ levels and PKA-mediated phosphorylation. At a given CL (which depends strictly on pacing frequency), APD/CL depends directly on the degree to which the dynamic net inward-outward current balance tips in the inward versus outward direction, thereby lengthening and shortening the APD, respectively. The degree of net tipping, in turn, is governed by the rates of inwardly versus outwardly conducting ion channel activation versus inactivation/deactivation state transitions. APD per se, is governed largely by the Kir2.1 activation rate, which, in turn, depends on the rate at which $\Delta\psi_m(t)$ repolarizes to < -50 mV (governed by $d(\Delta\psi_m(t))/dt$ during AP phase 3).

Anomalous decrease of APD/CL due to severe bradycardia and/or APD shortening beyond nominal limits promotes I_{Ca,L} buildup to non-paced depolarizing levels. Conversely, anomalous increase of APD/CL due to severe tachycardia and/or APD expansion beyond nominal limits promotes collisions between successive paced APs (described by Fossa [22] as the loss of restitution time). Hondeghem et al. proposed that pro-arrhythmic versus non-pro-arrhythmic hERG blockade are distinguishable based on a set of criteria consisting of triangulation of the AP morphology, reverse use dependence of APD, instability, and dispersion of repolarization (“TRIaD”) [23]. Although our findings suggest that pro-arrhythmic propensity is governed solely by dynamic fractional ion channel occupancy (not necessarily limited to hERG), the TRIaD concept can be viewed as the phenotypic counterpart of our proposed pro-arrhythmia criteria, as follows:

- 1) Perturbation-induced changes in AP morphology (not necessarily triangulation). Our findings suggest that AP lengthening follows from decreased $d(\Delta\psi_m(t))/dt$ in the shoulder region on approach to T₁. APD prolongation is graded as a function of perturbation level, resulting in isomorphic (putatively anti-arrhythmic) and non-

isomorphic (certainly pro-arrhythmic) variations at smaller versus larger prolongation levels, respectively. Our results suggest that therapeutic APD prolongation by class III anti-arrhythmic drugs necessarily falls within a Goldilocks zone between anti-arrhythmic increases in refractoriness versus pro-arrhythmic increases in APD/CL, and concomitant loss of restitution time. Our simulations clearly suggest that far greater levels of hERG blockade are tolerable than is widely assumed, consistent with the existence of a therapeutic index for class III hERG blocking anti-arrhythmic agents.

- 2) The susceptibility of $\text{Ca}_v1.2$ to undergo spontaneous recovery from inactivation at slower HR/longer CL is consistent with reverse use dependence.
- 3) Instability, corresponding to oscillations in $d(\Delta\psi_m(t))/dt$ and $\text{Ca}_v1.2$ recovery within the PT_1 regime.
- 4) Dispersion, corresponding to differential effects of perturbation on APDs in M, epi- and endocardial cells, manifesting as increased wavelength of the T wave.

Conclusion

We used ORD/hERG Markov to simulate the progression of normal cardiac APs to the arrhythmic state under conditions of hERG blockade/loss of function and $\text{Na}_v1.5$ gain of function, focusing on the underlying ion current anomalies driving the process. We describe the AP generation mechanism on the basis of Biodynamics theory (dynamic inward-outward current balance optimized for stimulus-response based electrical behavior and excitation-contraction coupling) and arrhythmogenesis (anomalous dynamic inward-outward current balance that promotes spontaneous depolarizations and AP-AP collisions). The major findings can be summarized as follows:

- 1) Arrhythmogenesis (irrespective of etiology) may be partitioned into graded pro-arrhythmic, pre-arrhythmic, and arrhythmic regimes as a function of perturbation severity and time, such that abrupt inter-regime transitions occur over tiny perturbation increments (somewhat analogous to buffered acid-base titration).
- 2) All phases of arrhythmogenesis depend on pacing CL. Longer CL/slower heart rates promote spontaneous atypical $\text{I}_{\text{Ca,L}}$ -driven depolarizations (specifically due to anomalous recovery from the CDI and VDI states of $\text{Ca}_v1.2$), whereas shorter CLs promote collisions between successive APs that result in pacing-induced $\text{I}_{\text{Ca,L}}$ -driven depolarizations. Arrhythmogenesis is thus governed by interactions between paced and non-paced APs (Figures 32-35). Arrhythmic AP wavelengths expand via collisions between spontaneous non-paced and paced depolarizations (noting that collision frequency grows with increasing wavelength above the pacing CL). This process culminates in continuous oscillations spanning across the diastolic interval of each cycle.

- 3) The AP generation system responds to increasing perturbation levels in a quasi-linear fashion throughout the pro-arrhythmic regime (Figure 24). However, non-linear progression to the arrhythmic state is tacitly assumed under the current hERG blocker safety assessment protocol. Our results reiterate our previous claim that significantly greater exposure is required to achieve pre-arrhythmic occupancy for non-trappable blockers exhibiting slower k_{on} , versus trappable blockers or non-trappable blockers with faster k_{on} (at equivalent IC_{50}) (Figure 23). Revision of the hERG safety margin may be warranted based on our simulated mechanism-based dose-response results (further addressed in [18]), which furthermore, could facilitate more straightforward consideration of benefit/risk criteria in the hERG safety equation.

References

1. Pearlstein RA, Wan H, Aravamuthan V. Toward in vivo relevant drug design. *Drug Discov Today*. Elsevier Ltd; 2020;00. doi:10.1016/j.drudis.2020.10.012
2. Baker DW, Einstadter D, Thomas C, Cebul RD. Mortality trends for 23,505 Medicare patients hospitalized with heart failure in Northeast Ohio, 1991 to 1997. *Am Heart J*. 2003;146: 258–264. doi:10.1016/S0002-8703(02)94784-8
3. Huikuri H V., Castellanos A, Myerburg RJ. Sudden Death Due to Cardiac Arrhythmias. *N Engl J Med*. Massachusetts Medical Society ; 2001;345: 1473–1482. doi:10.1056/NEJMra000650
4. Weiss JN, Garfinkel A, Karagueuzian HS, Chen PS, Qu Z. Early afterdepolarizations and cardiac arrhythmias. *Hear Rhythm*. Elsevier Inc.; 2010;7: 1891–1899. doi:10.1016/j.hrthm.2010.09.017
5. Gilmour RF. Early afterdepolarization-induced triggered activity: Initiation and reinitiation of reentrant arrhythmias. *Heart Rhythm*. 2004. pp. 449–450. doi:10.1016/j.hrthm.2004.07.005
6. Pogwizd SM, Schlotthauer K, Li L, Yuan W, Bers DM. Arrhythmogenesis and contractile dysfunction in heart failure: Roles of sodium-calcium exchange, inward rectifier potassium current, and residual beta-adrenergic responsiveness. *Circ Res*. 2001;88: 1159–67.
7. Shannon TR, Pogwizd SM, Bers DM. Elevated Sarcoplasmic Reticulum Ca²⁺ Leak in Intact Ventricular Myocytes From Rabbits in Heart Failure. *Circ Res*. 2003;93: 592–594.

doi:10.1161/01.RES.0000093399.11734.B3

8. Christiaans I, Elliott PM. Hypertrophic cardiomyopathy. *Clinical Cardiogenetics: Second Edition*. 2016. pp. 61–74. doi:10.1007/978-3-319-44203-7_4

9. Coppini R, Ferrantini C, Yao L, Fan P, Del Lungo M, Stillitano F, et al. Late sodium current inhibition reverses electromechanical dysfunction in human hypertrophic cardiomyopathy. *Circulation*. 2013;127: 575–584.
doi:10.1161/CIRCULATIONAHA.112.134932

10. Myles RC, Wang L, Bers DM, Ripplinger CM. Decreased inward rectifying K⁺ current and increased ryanodine receptor sensitivity synergistically contribute to sustained focal arrhythmia in the intact rabbit heart. *J Physiol*. 2015;593: 1479–1493.
doi:10.1113/jphysiol.2014.279638

11. O’Hara T, Virág L, Varró A, Rudy Y. Simulation of the undiseased human cardiac ventricular action potential: model formulation and experimental validation. *PLoS Comput Biol*. 2011;7: e1002061. doi:10.1371/journal.pcbi.1002061

12. Pearlstein RA, MacCannell KA, Erdemli G, Yeola S, Helmlinger G, Hu Q-Y, et al. Implications of Dynamic Occupancy, Binding Kinetics, and Channel Gating Kinetics for hERG Blocker Safety Assessment and Mitigation. *Curr Top Med Chem*. 2016;16: 1792–1818. doi:10.2174/1568026616666160315142156

13. Pearlstein RA, Dickson CJ, Hornak V. Contributions of the membrane dipole potential to the function of voltage-gated cation channels and modulation by small molecule potentiators. *Biochim Biophys Acta - Biomembr*. Elsevier; 2017;1859: 177–194.

doi:10.1016/J.BBAMEM.2016.11.005

14. Rudy Y, Hara TO. Simulation of the Undiseased Human Cardiac Ventricular Action Potential : Model Formulation and Experimental Validation. 2011;7.
doi:10.1371/journal.pcbi.1002061
15. Redfern WS, Carlsson L, Davis AS, Lynch WG, MacKenzie I, Palethorpe S, et al. Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: Evidence for a provisional safety margin in drug development. Cardiovasc Res. 2003;58: 32–45. doi:10.1016/S0008-6363(02)00846-5
16. Grant AO. Cardiac ion channels [Internet]. Circulation: Arrhythmia and Electrophysiology. Lippincott Williams & Wilkins; 2009. pp. 185–194.
doi:10.1161/CIRCEP.108.789081
17. O’Hara T, Virág L, Varró A, Rudy Y, Hara TO, O’Hara T, et al. Simulation of the undiseased human cardiac ventricular action potential: model formulation and experimental validation. PLoS Comput Biol. 2011;7: e1002061.
doi:10.1371/journal.pcbi.1002061
18. Wan H, Selvaggio G, Pearlstein R. Toward in vivo -relevant hERG safety assessment and mitigation strategies based on relationships between non-equilibrium blocker binding, three-dimensional channel-blocker interactions, dynamic occupancy, dynamic exposure, and cellular arrhythmia. Silman I, editor. PLoS One. Public Library of Science; 2020;15: e0234946. doi:10.1101/2020.06.08.139899

19. Selvaggio G, Pearlstein RA. Biodynamics: A novel quasi-first principles theory on the fundamental mechanisms of cellular function/dysfunction and the pharmacological modulation thereof. *PLoS One*. 2018;13: e0202376. doi:10.1371/journal.pone.0202376

20. Baltogiannis GG, Johnson DM, Verkerk AO, Wilders R. Long QT Syndrome and Sinus Bradycardia-A Mini Review. *Front Cardiovasc Med* | www.frontiersin.org. 2018;1: 106. doi:10.3389/fcvm.2018.00106

21. Pearlstein RA, McKay DJJ, Hornak V, Dickson C, Golosov A, Harrison T, et al. Building New Bridges between In Vitro and In Vivo in Early Drug Discovery: Where Molecular Modeling Meets Systems Biology. *Curr Top Med Chem*. 2017;17: 1–1. doi:10.2174/1568026617666170414152311

22. Fossa AA. Beat-to-beat ECG restitution: A review and proposal for a new biomarker to assess cardiac stress and ventricular tachyarrhythmia vulnerability. *Ann Noninvasive Electrocardiol*. 2017;22: 1–11. doi:10.1111/anec.12460

23. L.M. Hondeghem, MD P, L. Carlsson P, G. Duker P. Instability and Triangulation of the Action Potential Predict Serious Proarrhythmia, but Action Potential Duration Prolongation Is Antiarrhythmic. *Circulation*. 2001;103: 2004–2013. doi:10.1161/01.CIR.103.15.2004