Revealing the Concealed Nature of Long-QT Type 3 Syndrome

Amara Greer-Short, PhD; Sharon A. George, PhD; Steven Poelzing, PhD; Seth H. Weinberg, PhD

Background—Gain-of-function mutations in the voltage-gated sodium channel (Nav1.5) are associated with the long-QT-3 (LQT3) syndrome. Nav1.5 is densely expressed at the intercalated disk, and narrow intercellular separation can modulate cell-to-cell coupling via extracellular electric fields and depletion of local sodium ion nanodomains. Models predict that significantly decreasing intercellular cleft widths slows conduction because of reduced sodium current driving force, termed “self-attenuation.” We tested the novel hypothesis that self-attenuation can “mask” the LQT3 phenotype by reducing the driving force and late sodium current that produces early afterdepolarizations (EADs).

Methods and Results—Acute interstitial edema was used to increase intercellular cleft width in isolated guinea pig heart experiments. In a drug-induced LQT3 model, acute interstitial edema exacerbated action potential duration prolongation and produced EADs, in particular, at slow pacing rates. In a computational cardiac tissue model incorporating extracellular electric field coupling, intercalated cleft sodium nanodomains, and LQT3-associated mutant channels, myocytes produced EADs for wide intercellular clefts, whereas for narrow clefts, EADs were suppressed. For both wide and narrow clefts, mutant channels were incompletely inactivated. However, for narrow clefts, late sodium current was reduced via self-attenuation, a protective negative feedback mechanism, masking EADs.

Conclusions—We demonstrated a novel mechanism leading to the concealing and revealing of EADs in LQT3 models. Simulations predict that this mechanism may operate independent of the specific mutation, suggesting that future therapies could target intercellular cleft separation as a compliment or alternative to sodium channels. (Circ Arrhythm Electrophysiol. 2017;10:e004400. DOI: 10.1161/CIRCEP.116.004400.)

Key Words: action potential • edema • long QT syndrome • mutation • sodium channels

The long-QT type 3 (LQT3) syndrome is associated with gain-of-function mutations in the gene encoding the cardiac voltage-gated sodium (Na+) channel (Nav1.5). In isolated myocytes, this gain-of-function generates a late Na+ current that reproducibly triggers early afterdepolarizations (EADs).1,2 However, LQT3 can also be a concealed disease,3,4 requiring some other pathogenesis to unmask it, consistent with the relative difficulty of producing EADs in intact tissue.5,6 The observation that LQT3-associated EADs are easier to produce in isolated myocytes than tissue has led to the hypotheses that gap junction coupling reduces action potential (AP) dispersion and prevents EAD propagation,7 presumably by increased current sink into neighboring nondepolarizing myocytes. Simulations suggest that reduced gap junction coupling can promote EAD formation in tissue, by reducing the total number of EAD-producing myocytes necessary for EAD propagation.7 However, the main experimental evidence to support this mechanism is the fact that EADs are more rare in tissue preparations.

Recently, we and others demonstrated that Nav1.58–13 and Na+ current14,15 are enhanced in the intercalated disk (ID). Furthermore, we suggested that the narrow intercellular separation in Nav1.5-rich nanodomains could modulate electric coupling via localized extracellular ion-mediated effects, also termed ephaptic coupling (EpC).16–18 Although there are multiple proposed mechanisms of EpC,18 this study focuses on 2 mechanisms: (1) extracellular electric fields: Na+ channels in the depolarizing cell decrease the ID cleft potential, depolarizing the apposing membrane from the extracellular rather than the intracellular domain and (2) local Na+ ion depletion: dynamic alteration of Na+ concentration in the extracellular cleft alters the driving force of individual Na+ ions.

Importantly, computational models of EpC suggest that conduction velocity (CV) is biphiscally related to EpC.10,16,17,19 In tissue with narrow intercellular cleft separations, conduction is modulated by a phenomenon termed “self-attenuation,”10,20 whereby decreased intercellular separation slows conduction by two mechanisms: (1) an increase in downstream transmembrane potential at the ID ($V_{m}^{ID}$) and

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WHAT IS KNOWN

- The LQT3 syndrome is a concealed disease that is associated with gain-of-function mutations in the gene encoding the cardiac voltage-gated sodium channel.
- This gain-of-function generates a late sodium current that consistently triggers arrhythmogenic EADs in isolated myocytes. However, EADs are harder to reproduce in cardiac tissue.

WHAT THE STUDY ADDS

- In computational and experimental LQT3 models, increasing intercellular separation in sodium channel-rich ID nanodomains can prolong APs and promote EADs, whereas narrow intercellular separation can conceal the LQT3 phenotype and suppress EADs.
- LQT3 masking occurs because of negative feedback from the late sodium current localized at the ID that depletes the sodium concentration in the intercellular cleft.

(2) Na+ ion depletion from the intercellular cleft, reducing the sodium reversal potential ($E_{\text{Na}}^{\text{dis}}$). Both mechanisms attenuate Na+ current ($I_{\text{Na}}^{\text{disc}}$) driving force, reducing peak $I_{\text{Na}}^{\text{dis}}$, and thus reduce CV.

In this study, we tested the novel hypothesis, both ex vivo and in silico, that the concealed nature of LQT3 in intact tissue can arise from self-attenuation. Critically, in the concealed phase of the disease, despite conductive Na+ channels, late $I_{\text{Na}}^{\text{dis}}$ is significantly reduced because of attenuated driving force, suppressing EADs.

Methods

All animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Virginia Polytechnic University. Optical voltage mapping of Langendorff perfused hearts has been extensively described by the Poelzing laboratory. Full details of the computational model are given in the Methods section in the Data Supplement. In brief, the anterior epicardial surfaces of the left and right ventricles from retired breeder guinea pigs were mapped during pacing. Late Na+ current agonist, sea anemone venom ATXII (7 nmol/L), was perfused into hearts at a concentration previously demonstrated to prolong AP duration (APD), without apparently producing EADs in guinea pigs. Mannitol (26.1 g/L) was perfused to produce acute interstitial edema (AIE) 24 and 25,26 on 3 hearts per treatment. Additional details can be found in the Methods section in the Data Supplement.

The total number of experiments for each intervention was as follows: control 12, mannitol 5, ATXII 7, ATXII and mannitol 7. Data are presented as mean±SE unless otherwise noted, and $P<0.05$ was considered statistically significant.

Results

Ex Vivo

The focus of our study was to investigate arrhythmogenic consequences of modulating ID width in an LQT3 model of cardiac tissue. We first tested the hypothesis that ID expansion can prolong APD and unmask EADs in a drug-induced, guinea pig model of LQT3. Representative transmission electron microscopy micrographs and summary data from a blinded observer demonstrated that intercellular separation ($w_p$) within the perinexus (Figure 2A) with ATXII was 18.3±0.7 nm (95% confidence interval [CI], 17.0–19.6 nm, blue), similar to previously published measurements in murine and guinea pig ventricular myocardium estimated by the same observer with similar perfusate compositions. Mannitol significantly expanded $w_p$ to 24.±0.7 nm (95% CI, 23.1–25.7 nm, AIE, green; $P=0.003$). Importantly, $w_p$ was similar between AIE and ATXII+AIE: ATXII (23.9±0.6 nm; 95% CI, 22.7–25.1 nm, red) hearts. These data demonstrate that ATXII does not measurably alter nanodomain expansion secondary to mannitol-induced AIE.

Optical mapping experiments revealed that increasing $w_p$ with mannitol alone (Figure 2B, AIE, green) did not significantly change APD90, regardless of cycle length (CL) relative to control (Figure 2B; Table III in the Data Supplement, black; $P=0.141$, at CL=500 ms, 165±3 ms; 95% CI, 153–177 ms versus 177±10 ms; 95% CI, 152–203 ms, AIE versus control, respectively). When hearts were perfused with ATXII (blue) to pharmacologically induce LQT3, APD90 was statistically greater at multiple CLs, and the APD restitution curves diverged at greater CLs (Figure 2B; Table III in the Data Supplement, ATXII 249±7 ms; 95% CI, 231–266 ms). This can be seen more clearly in Figure 2C, where the proportional APD increase from ATXII to ATXII+AIE at CL=160 ms was 6±1% (95% CI, 5%–8%). Increasing the CL to 600 ms further and significantly increased this proportionality relative to 160 ms ($P=0.0494$, 30±12%; 95% CI, 8%–38%). In addition, a regression analysis revealed that ATXII and ATXII+AIE significantly increase APD restitution slope, compared with...
control, whereas there is no difference for AIE alone (Table IV in the Data Supplement).

We further assessed arrhythmic burden in our guinea pig model of LQT3 by quantifying the proportion of hearts that had at least 1 R-on-T event during the last 20 minutes of the intervention, determined by a QRS complex occurring at a time corresponding to the last nonectopic beat’s T-wave measured from the volume conducted bath ECG (Figure 2D). R-on-T
Spatially restricted nature of intercellular clefts (Figure 2A). The high density of Na⁺ channels localized at the ID suggests that Na⁺-dependent signaling and demonstrates the need for computational modeling to account for the time-dependent dynamics of Na⁺ influx, efflux, and diffusion at the intercellular cleft, especially in pathological settings of Na⁺ channel mutations.

**In Silico**

We next performed simulations in a strand of myocytes that contain an LQT3-associated Na⁺ channel mutant (Y1795C) to identify the mechanism for EAD unmasking during intercellular cleft expansion and EAD suppression during cleft narrowing. Myocytes were paced at a 1000-ms CL at one end of the strand. Figure 3 shows the transmembrane potential ($V_m^\text{disk}$), Na⁺ channel open probability ($p_{\text{open},\text{Na}}^\text{disk}$), Na⁺ current ($I_{\text{Na}}^\text{disk}$), and Na⁺ reversal potential ($E_{\text{Na}}^\text{disk}$) at the upstream ID membrane patches in cells near the strand end (cells 10 and 40). Traces at disk patches from only 2 cells are shown for clarity; space-time $V_m^\text{disk}$ plots are shown in the Results section in the Data Supplement (Figure 1 in the Data Supplement).

Wild-type (WT) cells did not produce EADs in strands with either narrow ($w=10$ nm, dashed black) or wide ($w=50$ nm, green) clefts (Figure 3A), and there was no difference between APDs. APD was prolonged with Y1795C mutant myocytes in strands with both narrow (blue) and wide clefts (red). However, even with the mutation, myocytes in narrow cleft strands did not generate EADs, whereas mutant myocytes in wide cleft strands produced EADs on every other beat.

**Sodium Channel Open Probability, Current, and Reversal Potential**

To explore the theoretical mechanisms of EAD formation in strands, Na⁺ channel open probability, current, and reversal potential were tracked during the AP. $p_{\text{open},\text{Na}}^\text{disk}$ increased with the Y1795C mutation in narrow cleft strands as expected (Figure 3B, blue). As the AP repolarized, the late $I_{\text{Na}}^\text{disk}$ increased (Figure 3C, blue). Importantly, we observed interesting dynamics that prevented EAD formation in narrow cleft strands. Narrow cleft strands supported EpC, promoting 2 critical phenomena: first, $E_{\text{Na}}^\text{disk}$ was reduced in mutant narrow cleft strands immediately after the AP upstroke because of depleted [Na⁺]e (Figure 3D, blue, arrow 1), which decreased the late $I_{\text{Na}}^\text{disk}$ compared with mutant wide cleft strands (Figure 3C, red). After the AP upstroke, [Na⁺]e began to refill, such that $E_{\text{Na}}^\text{disk}$ began to increase (arrow 2). Second, critically, the late $I_{\text{Na}}^\text{disk}$ promoted a second depletion of [Na⁺]e (arrow 3), which decreased $E_{\text{Na}}^\text{disk}$ further. This protective negative feedback mechanism decreased the late $I_{\text{Na}}^\text{disk}$ via ionic self-attenuation, caused by [Na⁺]e depletion. The end result was that APD in mutant narrow cleft strands was longer than in WT strands, but EADs did not form.
In contrast, increasing cleft width produced EADs in mutant strands because ionic self-attenuation did not occur to the same extent. Similar to narrow cleft strands, both $p_{\text{open, Na}}$ and $I_{\text{Na}}$ were increased in mutant wide cleft strands (Figures 3B and 3C, red). However, late $I_{\text{Na}}$ amplitude was larger. Wide clefts reduced EpC effects, and as a result, $[\text{Na}^+]_{\text{e}}$ was not depleted to the same extent as occurred in narrow cleft strands. In short, $E_{\text{Na}}$ disk was larger in the mutant wide cleft width strands immediately after the AP upstroke (Figure 3D, red). The end result was that late $I_{\text{Na}}$ significantly increased in mutant wide strands, promoting EADs. The model, therefore, predicts that the loss of negative feedback via EpC maintains $[\text{Na}^+]_{\text{e}}$ elevated to promote EADs. Thus, we hypothesize that narrow clefts suppress EADs in LQT3 by EpC-negative feedback mechanisms caused by $[\text{Na}^+]_{\text{e}}$ depletion in the ID where Na+ channels are densely expressed.

It is important to note that EADs occurred on every other beat. Supporting results (Figure II in the Data Supplement) demonstrate that this was a consequence of Na+ channel burst mode open state refractoriness.

APD and Cleft Width
APD is shown as a function of cleft width in WT (black) and mutant (red) strands during 1000-ms CL pacing in Figure 4A. In WT strands, APD did not depend on cleft width. However, in mutant strands, EADs were unmasked for cleft widths >20 nm. APD increased as cleft width increased, with “jumps” at irregular intervals, corresponding to increasingly longer EADs.

APD and CL
The above experiments only discussed the mechanisms of EAD suppression and formation at a 1000-ms CL. However, bradycardia is known to exacerbate arrhythmogenic risk in LQ3. Therefore, we explored the CL-dependence of APD as well (Figure 4B). In WT strands, APD gradually increased as CL increased, with negligible difference between wide (green) and narrow (black) strands, consistent with typical APD restitution. In narrow mutant strands (Figure 4B, blue), APD was slightly longer compared with WT, and only at very long CLs.
more than ≈1700 ms were EADs unmasked. EAD formation at very long CLs in mutant narrow cleft strands occurs via increased [Na]$^{\text{disk}}_{\text{e}}$ refilling after long diastolic intervals because of both pump and exchanger activity and extracellular diffusion while the Na+ channels are nonconductive.

In wide mutant strands (red), at shorter CLs less than ≈900 ms, APD was also longer compared with narrow mutant strands, but EADs did not form because [Na]$^{\text{disk}}_{\text{e}}$ was insufficient to sustain the elevated $E_{\text{INa}}^{\text{disk}}$ required for significant late $I_{\text{INa}}^{\text{disk}}$. However, for longer CLs more than ≈900 ms, EADs were unmasked, and APD increased greatly as CL increased. Importantly, the model predicts that modulating [Na]$^{\text{disk}}_{\text{e}}$ accumulation may be a new mechanism underlying EAD formation in LQT3, independent of the mutant channel kinetics.

**Conduction and Cleft Width**

Although the relationship between conduction and intercellular separation within the ID is the subject of numerous computational studies, this relationship has not been explored for Na+ channel gain-of-function mutations. CV is shown as a function of cleft width in WT (black) and mutant (red) strands during 1000-ms CL pacing (Figure 4C). In both WT and mutant strands, CV had a biphasic dependence. For cleft widths between 10 and 50 nm, CV decreased as cleft width increased, as EpC was reduced, consistent with our previous experimental data. For narrow clefts, CV decreased as cleft width decreased because of electric field–induced self-attenuation, also consistent with our recent experimental report. The model predicts the Y1795C mutation slightly increased CV relative to WT at clefts widths >20 nm and <10 nm. However, in the nominal cleft width range found in guinea pig and mouse ventricular tissue (10–20 nm), CV is predicted to be relatively similar for WT and mutant strands.

**Conduction and CL**

The predicted relationship between CV and CL is equally interesting (Figure 4D). Relative to wide cleft strands (Figure 4D, red and green), CV restitution in narrow strands (blue and black) was broader, that is, CV decreased over a wider range of CLs. In particular, CV at CLs <500 ms was slower in narrow cleft strands (black and blue) than in wide cleft strands (green and red). This finding did not depend on the type of sodium channel (WT or Y1795C). Yet, the opposite occurred at higher CLs >500 ms, where CV was greater in the narrow (black and blue) relative to wide cleft strands (green and red), as in Figure 4C, CV was reduced to a greater extent at short CLs in narrow strands because electric field–induced self-attenuation of peak $I_{\text{INa}}^{\text{disk}}$ is greater, relative to longer CLs.

**Ephaptic Electric Field Coupling vs Cleft Sodium**

Next, we ran simulations to determine to what extent extracellular electric field coupling and [Na]$^{\text{disk}}_{\text{e}}$ depletion separately influenced EAD masking in narrow cleft mutant strands paced at a 1000-ms CL (Figure 6). We found that clamping [Na]$^{\text{disk}}_{\text{e}}$ to the bulk extracellular sodium ([Na]$^{\text{b}}_{\text{e}}$) concentration created EADs for all cleft widths (Figure 6A and 6B, green), demonstrating that in narrow strands with dense Na+ channel localization, [Na]$^{\text{disk}}_{\text{e}}$ depletion is critical to the ionic self-attenuation mechanism that reduced the late $I_{\text{INa}}^{\text{disk}}$ and masked EADs. To probe the contribution of electric field coupling, the extracellular T-shaped resistor network (Figure 1A) was removed such that an extracellular potential could not develop at the ID, that is, $\phi_{\text{EpC}}^{\text{cleft}}$ and $\phi_{\text{EpC}}^{\text{disk}}$ were fixed at 0. When electric field coupling was removed (red), EADs were still masked in narrow cleft width strands. APD in mutant myocyte strands with (blue) and without electric field coupling (red) were close for all cleft widths (Figure 6B). However, without electric field coupling, CV monotonically increased as a function of cleft width (Figure 5C, red), further supporting previous conclusions that electric field coupling mediates the CV decrease at wider cleft widths. Both clamping [Na]$^{\text{disk}}_{\text{e}}$ and removing electric field coupling resulted in no CV dependence on cleft width (magenta), as EADs were unmasked and CV was a constant for all cleft widths.

We performed several additional simulations to identify the critical factors necessary for concealing EADs in LQT3. To match the CV decrease previously observed in experiments as cleft width increases, the gap junction conductance value from the Kucera et al. EpC model was reduced. Importantly, we found that the negative feedback mechanism for EAD suppression did not depend on gap junction conductance, as reduced gap junctional coupling only slightly
altered the APD dependence on cleft width (Figure III in the Data Supplement). Next, we found that high Na channel 1D localization was required to mask EADs in narrow cleft strands because lateral membrane localized Na channels, exposed to an invariant \([Na^{+}]_{e}\), did not drive the negative feedback necessary to suppress the late Na current (Figure IV in the Data Supplement). Furthermore, we showed that EADs were suppressed in a myocyte strand with nonjunctonal extracellular spaces, consistent with \([Na^{+}]_{e}^{\text{cleft}}\) depletion simulations in Figure 6; however, EADs were masked over a smaller extracellular volume range in myocyte strands with nonjunctonal extracellular spaces, compared with cleft spaces because of the absence of high Na channel junctonal localization (Figure V in the Data Supplement). Parameter studies varying the transition rates in the sodium channel Markov model showed that the transition rates between the “background mode” and “burst mode” were primarily responsible for prolonging APD and promoting EADs in myocyte strands with wide clefts (Figure VI in the Data Supplement).

Finally, simulations demonstrated that the protective mechanism underlying EAD masking in strands with narrow cleft width was similarly robust in a different LQT3-associated sodium channel mutant (I1768V; Figure VIIA in the Data Supplement) and different species (canine, Figures VII B and VII C in the Data Supplement). Importantly, the mechanism underlying the late Na current in the 2 mutant types are distinct: Y1795C mutant channels fail to inactivate\(^27\) and I1768V mutant channels exhibit a faster inactivation recovery that facilitates channel reopening\(^28\); neither mutant channel presents with steady-state channel reopening nor “window current.” Thus, simulations demonstrated that masking EADs did not depend on the specific molecular mechanism underlying the late Na current.
Discussion

In this study, we demonstrated a novel mechanism leading to the formation of EADs in the long-QT type 3 syndrome, findings supported by a guinea pig acquired LQT3 model. This new mechanism may operate independent of the type of specific mutation associated with LQT3 and the underlying channel dysfunction producing the late Na\(^+\) current. Both experiments and simulations demonstrated that increasing intercellular separation in Na\(^+\) channel–rich ID nanodomains can exacerbate APD prolongation in LQT3, leading to arrhythmogenic EADs. The proposed mechanism by which narrow intercellular clefts conceal LQT3 is related to the depletion of Na\(^+\)\(_{cleft}\), an ionic self-attenuation mechanism. In summary, \(I_{Na}^{kin} \text{ reduces } E_{Na}^{kin} \text{ via the depletion of the local } [Na^+]_{cleft}, \text{ which, in turn, reduces } I_{Na}^{kin} \text{ via a negative feedback mechanism. Taken together, the experimental and computational results suggest that bradycardia and extracellular volume expansion in the ID can unmask an arrhythmogenic LQT3 phenotype, whereas an LQT3 mutation or ID expansion individually only produce modestly abnormal electrophysiological behavior.}

Previous computational modeling studies have provided valuable insights into the cellular mechanisms, leading to APD prolongation and EADs during bradycardia in myocytes with LQT3-associated mutations.\(^{2,27,28}\) Clancy et al.\(^{37}\) demonstrated that the amount of time mutant channels spend bursting (burst mode dwell time) may be the primary determinant of the inverse heart-rate dependence of the sustained late Na\(^+\) current. At fast heart rates, despite the presence of a large late Na\(^+\) current, relative to WT myocytes, APD was minimally prolonged because of incomplete deactivation of the slow-component of the delayed rectifier K\(^-\) channel \(I_{Ks}\). At slower heart rates, sufficient time between beats allowed for complete deactivation of \(I_{Ks}\), shifting the balance of depolarizing and repolarizing currents and significantly prolonging APD. Furthermore, simulations have illustrated that while the late Na\(^+\) current triggers EADs, the depolarizing current driving EADs is carried by the L-type Ca\(^{2+}\) channel.\(^{19}\) The late Na\(^+\) current prolongs the AP sufficiently long to allow for recovery from inactivation and reactivation of the L-type Ca\(^{2+}\) channel.

Simulations of therapies directly targeting the Na\(^+\) channel have been used to predict the efficacy of various pharmacological treatments.\(^{34-38}\) Vecchietti et al.\(^{39}\) illustrated that reducing Na\(^+\) and Ca\(^{2+}\) current conductances suppressed EADs in LQT3-associated mutant myocytes. More recent studies have highlighted the importance of drug binding kinetics, including drugs with state-specific binding sites. Clancy et al.\(^{40}\) predicted that open-state channel blockers are more efficacious in LQT3-associated \(\Delta KPQ\) mutant channels, which have longer channel mean open times due to abnormal burst mode gating, compared with WT channels. In contrast, inactivated-state channel blockers are less effective in suppressing EADs and may adversely affect conduction by reducing peak \(I_{Na_s}\). Moreno et al.\(^{41}\) performed an in silico screening of ranolazine in myocytes with \(\Delta KPQ\) mutant channels. Ranolazine not only exhibits preferential block of late Na\(^+\) current relative to peak Na\(^+\) current but also blocks the rapid component of the delayed rectifier K\(^-\) current \(I_{K_s}\). Simulations predicted that ranolazine suppresses bradycardia- and pause-induced EADs and does not reduce CV, findings independent of the specific myocyte ionic model used in the simulation.

Computational studies have also played an integral role on elucidating arrhythmogenic mechanisms in the interactions between intracellular signaling cascades and Na\(^+\) channel regulation. Simulation of \(\beta\)-adrenergic agonists in myocyte strands with \(\Delta KPQ\) mutant channels was predicted to suppress EADs and reduce transmural dispersion of repolarization because of increased peak \(I_{K_s}\) and \(I_{Kr}\). However, the \(\beta\)-blocker propranolol, in combination with isoproterenol, was predicted to prolong APD in low doses and shorten APD in high doses, suggesting the need for dose-controlled clinical trials for LQT3 patients using \(\beta\)-blockers.\(^{38}\) A recent study by Koval et al.\(^{37}\) found that 2 different SCN5A mutants led to aberrant phosphorylation by Ca\(^{2+}\)/calmodulin-dependent protein kinase II, enhancing the late Na\(^+\) current and increasing susceptibility to EADs.

Importantly, while many of the aforementioned simulation studies were performed in 1-, 2-, or 3-dimensional tissues (however, neglecting EpC and cleft Na\(^+\) nanodomains), the mechanism underlying EAD formation involved the balance of Ca\(^{2+}\), K\(^-\), and Na\(^+\) currents at the level of the individual myocytes. Furthermore, drug therapies typically targeted specific ionic currents: suppressing the late Na\(^+\) current by reducing channel conductivity or the channel open probability via increased dwell-time in nonconductive drug-bound state(s) or augmenting compensatory repolarizing currents via activation of intracellular signaling cascades. Critically, our results do not contradict the findings or interpretations discussed above. Instead, we provide an additional important mechanism that explains why EADs form particularly at slow heart rates in tissue, and we provide evidence that nanodomain cleft [Na\(^+]\) modulation is a novel mechanism to unmask EAD formation at these slow heart rates. Taken together, our current work and previous studies suggest that LQT3 patients may be at highest risk of lethal ventricular arrhythmias under conditions associated with bradycardia and extracellular edema as occurs with cardiac surgery,\(^{40}\) ischemia,\(^{40}\) or heart failure.\(^{41}\) Furthermore, future therapies aimed at reducing lethal arrhythmias in LQT3 could target reducing extracellular volume, a novel therapeutic approach that reduces late Na\(^+\) current in the ID but does not alter Na\(^+\) channel gating or activate intracellular signaling cascades (with potentially many off-target effects), as a complement or alternative to Na\(^+\) channel or other targeting therapies.

EpC in cardiac tissue has been investigated in previous computational studies\(^{10,16,17,19,32}\) and recently by us experimentally.\(^{10,20,24-26,42}\) These studies have typically focused on how EpC influences conduction and is modulated by gap junctional coupling, extracellular ionic concentrations, and other factors. Our findings agree with previous studies demonstrating that intercellular cleft width and Na\(^+\) channel localization are critical parameters regulating activation and conduction in cardiac tissue.\(^{16,17}\) To our knowledge, our current study is the first to demonstrate the importance of these properties in regulating repolarization in the setting of an arrhythmogenic mutation and further showing a potentially protective mechanism in which EpC, and in particular cleft Na\(^+\) depletion, can suppress EAD formation.
Limitations
Although our experimental results support computational predictions, the experiments are not without limitations. As with all studies relying on pharmacological agents, both ATXII and mannitol may have underestimated off-target effects individually and collectively. Experiments do not provide direct proof for intercellular cleft Na⁺ ion depletion. The observation that the experiments are well-described by a robust computational model is supporting evidence that incorporating extracellular electric field and cleft Na⁺ ionic coupling into a model can reproduce a diverse experimental data set, including the responses of cardiac conduction, APD restitution, and EAD formation to the late I_{to}, augmenting effects of ATXII and the perinuclear cleft expanding effects of mannitol. We do not claim that this constitutes proof that perinuclear cleft expansion is the primary mechanism of an unmasked LQT3 phenotype. Rather, models and experiments well describe each other, and these theories can generate new ideas to treat this complicated disease.

Although our computational model reproduces important aspects of electric conduction, it is not without limitations. Our model represents a 1-dimensional (1D) myocyte strand and thus does not account for the complex 3D structure of cardiac tissue. Furthermore, our model does not account for the 3D geometry of the individual myocyte, as in the electrodiffusion model of Mori et al. However, modeling Na⁺ channel localization and intracellular electrical propagation with a detailed biophysical ion model necessitated a numerical integration time step (0.05 μs) on the order of 1000x smaller than recent 2D and 3D tissue computational studies. As such, our model represents a compromise between computational cost and the level of subcellular and extracellular discretization necessary to account for critical biophysical details, as expanding to increasing dimensionality at either the cellular or tissue level would have been computationally challenging. In addition, model parameters for the cleft nanodomain were tuned to match experimental trends. Species differences between the sodium channel Markov models (fitted to patch clamp measurements of recombinant Na⁺ channels in human embryonic kidney 293 cells) and the ionic models (representing guinea pig and canine ventricular myocytes) may introduce differences between experimental and computational results. Finally, further studies are needed to determine to what extent ID localization of ion pumps, exchangers, and other ion channels and depletion or accumulation of other intercellular cleft ions (e.g., potassium, calcium, and chloride) influence EAD suppression during LQT3. Nonetheless, these limitations do not detract from the critical prediction of a novel mechanism underlying the concealment of the LQT3 phenotype.

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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL

Expanded Methods

Optical Mapping and Whole-Heart Experimental Details

Optical voltage mapping of Langendorff perfused guinea pig hearts has been extensively described by the Poelzing laboratory\textsuperscript{1-3}. Retired breeder guinea pigs were anesthetized with isoflurane inhalation and hearts were rapidly excised and retrogradely perfused with a modified Tyrode’s solution (in mM, CaCl\textsubscript{2} 1.25, NaCl 140, KCl 4.56, dextrose 5.5, MgCl\textsubscript{2} 0.7, HEPES 10; and 5.5 mL of NaOH per 1 L of Tyrode to pH to 7.4). Motion was reduced with 10 μM of blebbistatin. The anterior epicardial surface of the left and right ventricle were mapped during pacing by a bipolar plunge stimulating electrode placed on the right ventricular septum near the atrioventricular node at a current 1.5x the minimum stimulation threshold.

Sea anemone venom ATXII (7 nM), a late sodium current agonist\textsuperscript{4}, was perfused into hearts, because this concentration was previously demonstrated to prolong action potential duration (APD), without apparently producing EADs in a guinea pig model of pharmacologically induced LQT3\textsuperscript{5}. Mannitol at 26.1g/l was chosen to produce acute interstitial edema (AIE)\textsuperscript{6} and intercellular nanodomain expansion in the gap junction adjacent region of the intercalated disk called the perinexus\textsuperscript{1}. Henceforth we will simply refer to this condition as AIE recognizing that edema is a complex clinical pathology involving intra and extracellular volume expansion. The total number of optical mapping experiments was as follows: control (CTRL n=12 hearts), mannitol (AIE n=5), ATXII (n=7), ATXII and mannitol (ATXII+AIE n=7).

Hearts were paced at a cycle length of 160, 180, 200, 400, 500, 600, and 800 until loss of 1:1 capture. Action potential duration (APD) was measured as the difference from the time of the maximal rate of upstroke to 95% repolarization. APD at any given cycle length was only reported if all hearts in the experimental group could be paced 1:1 at that cycle length.

Data are reported as mean±standard error.

In Figure 2B, two statistical approaches were employed to determine whether an intervention altered APD restitution profiles. First, unpaired, unequal variance Student’s t-tests were performed to compare mean during an intervention to control and a Bonferroni correction of 4 was applied. A similar statistical analysis was used to compare ATXII+AIE to ATXII alone. Supplemental Table 3 contains mean, standard deviation, confidence intervals and p-values for APD measurements at all cycle lengths measured. A linear mixed effects (LME) regression model was applied using the statistical package R. Supplemental Table 4 contains mean, confidence intervals and p-values for this analysis.
In Figure 2C, the proportional change of APD (ΔAPD%) for ATXII + AIE was calculated as the APD difference of each heart for ATXII + AIE relative to the mean APD of ATXII alone. This difference was then normalized to the average of ATXII (for all hearts) to determine the relative change.

\[
\Delta APD\% = \frac{(APD_{ATXII + AIE, \text{individual heart}} - APD_{ATXII, \text{average of group}})}{APD_{ATXII, \text{average of group}}} \times 100
\]

The mean and standard error of ΔAPD% was then calculated for the 7 ATXII+AIE hearts. In order to calculate significance, we used an unpaired, unequal variance Student’s t-test to compare between ΔAPD% at CL=600 and 160ms. The purpose of this analysis was to determine whether the relative normalized APD difference for each cycle length were significantly different from 160ms to demonstrate that ATXII+AIE disproportionately prolongs APD at longer CL.

**Transmission Electron Microscopy Details**

In a subset of optical mapping experiments, tissue was fixed as previously described for TEM. Specifically, tissue from only the last 3 hearts in every experimental group was fixed for TEM: AIE, ATXII, ATXII+AIE. For each heart, 15 images were obtained by TEM. For each image, \( w_p \) was manually measured at 30, 45, 60, 75, 90 and 105nm, and these values were averaged to obtain \( w_p \) for each image. The average \( w_p \) for each heart was then the average of 15 \( w_p \) image measurements. Finally, the mean \( w_p \) presented in Figure 2A and associated standard error is based on 3 average \( w_p \) measurements in each experimental group. Statistical differences in the data set were first determined by ANOVA, and subsequently by two-sample equal variance t-tests with a Bonferroni correction of 2.

**Bath Electrocardiogram (ECG)**

Arrhythmic load was estimated from a volume conducted pseudo-ECG. In Figure 2D, the proportion of hearts with R on T phenomenon during AIE, ATXII or ATXII+AIE were each compared to control in 3 individual 2x2 Fisher’s Exact Tests, and a Bonferroni correction applied.

For all data, a \( p<0.05 \) after correction was considered statistically significant as is noted by symbols in the figures, while specific \( p \)-values are presented in the text.

**Computational Model Details**

The model for electrical conduction along a linear strand of 50 cells was modified from the ephaptic coupling model described by Kucera et al. The membrane of each cell was discretized into 10 axial patches and 2 disk patches, one at each end of the cell (Figure 1A). Each membrane patch generated currents proportional to the patch surface area: a capacitive current, with capacitance \( C_{\text{ax}} \) or \( C_{\text{disk}} \), and ionic currents \( I_{\text{ion}} \) governed
by the either Luo-Rudy dynamic (LRd) guinea pig\textsuperscript{11} or Hund-Rudy dynamic (HRd) canine\textsuperscript{12} ventricular myocyte model, modified as described below.

In the HRd model, the ionic current for each patch is given by the sum of the Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} currents carried by channels, pumps, and exchangers:

\[ I_{ion}^j = I_{Na,tot}^j + I_{K,tot}^j + I_{Ca,tot}^j, \]

where

\[
I_{Na,tot}^j = I_{Na}^j + I_{Na,b}^j + 3(I_{NCX}^j + I_{NCX,ss}^j) + I_{CaL,Na}^j + 3I_{NaK}^j,
\]

\[
I_{K,tot}^j = I_{Kr}^j + I_{Ks}^j + I_{K1}^j + I_{Kp}^j + I_{Cal,K}^j - 2I_{NaK}^j + I_{stim}^j,
\]

\[
I_{Ca,tot}^j = I_{CaL}^j + I_{Ca,b}^j + I_{pCa}^j + I_{CaT}^j - 2(I_{NCX}^j + I_{NCX,ss}^j).
\]

The currents are as follows: \( I_{Na} \) (fast Na\textsuperscript{+} current), \( I_{Na,b} \) (background Na\textsuperscript{+} current), \( I_{NCX} \) (intracellular Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger current), \( I_{NCX,ss} \) (subspace Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger current), \( I_{Cal,Na} \) (Na\textsuperscript{+} current carried by L-type Ca\textsuperscript{2+} channel), \( I_{NaK} \) (Na\textsuperscript{+}-K\textsuperscript{+} pump current), \( I_{Kr} \) (slow component of the delayed rectifier K\textsuperscript{+} current), \( I_{Ks} \) (slow component of the delayed rectifier K\textsuperscript{+} current), \( I_{K1} \) (inward rectifier K\textsuperscript{+} current), \( I_{Kp} \) (plateau K\textsuperscript{+} current), \( I_{Cal,K} \) (K\textsuperscript{+} current carried by L-type Ca\textsuperscript{2+} channel), \( I_{Cal} \) (Ca\textsuperscript{2+} current carried by the L-type Ca\textsuperscript{2+} channel), \( I_{Ca,b} \) (background Ca\textsuperscript{2+} current), \( I_{pCa} \) (Ca\textsuperscript{2+} pump current), \( I_{CaT} \) (T-type Ca\textsuperscript{2+} current), and \( I_{stim} \) (stimulus current).

In the LRd model, the ionic current for each patch is given by the sum of the Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}, and Cl\textsuperscript{2} currents carried by channels, pumps, and exchangers:

\[ I_{ion}^j = I_{Na,tot}^j + I_{K,tot}^j + I_{Ca,tot}^j + I_{Cl,tot}^j, \]

where

\[
I_{Na,tot}^j = I_{Na}^j + I_{Na,b}^j + 3(I_{NCX}^j + I_{NCX,ss}^j) + 3I_{NaK}^j,
\]
The currents are as above and as follows: $I_{Nall}$ (late Na$^+$ current), $I_{to1}$ and $I_{to2}$ (transient outward K$^+$ currents), and $I_{Clb}$ (background Cl$^-$ current).

In both models, the Hodgkin-Huxley type gating model for the $I_{Na}$ current was replaced with a 13-state Markov chain formulation developed by Clancy and colleagues$^{13}$, previously used to represent mutant Na$^+$ channels associated with LQT3 and producing a sustained late Na$^+$ current (Figure 1C).

Here, we consider models of two SCN5A mutations associated with the LQT3 phenotype: Y1795C$^{13}$ and I1768V$^{14}$. A common feature of both mutant channel models is two possible models of gating, a “background mode” and a “burst mode.” The background mode consists of 3 closed states, an open state, a fast inactivation state, 2 intermediate inactivation states, and 2 closed-inactivated states. The burst mode, consisting of 3 closed states and an open state, represent a population of channels that fail to inactivate.

In addition to accounting for the electric field effects via the cleft extracellular potential, we also accounted for localized depletion of cleft [Na$^+$]. The Na$^+$ reversal potential at the intercalated disk membrane patches was given by

$$E_{Na}^{disk} = \frac{RT}{z_{Na}F} \ln \left( \frac{[Na^+]_{cleft}^{cleft}}{[Na^+]_{disk}^{cleft}} \right),$$

where $z_{Na} = 1$ is the sodium ion valence, $F$ is Faraday’s constant, $R$ is the gas constant, and $T$ is the absolute temperature.

The extracellular sodium concentrations in the clefts between cells ([Na$^+$]$^{cleft}_e$) was governed by

$$\frac{d{[Na^+]_{e}^{cleft}}}{dt} = \frac{A_d \sum_{i=1}^{n} I_{Na}^{disk}}{z_{Na}F V_{cleft}} + \frac{[Na^+]_{e}^{bulk} - [Na^+]_{e}^{cleft}}{\tau_{Na}^{cleft}},$$

where the bulk extracellular sodium concentration ([Na$^+$]$^{bulk}_e$) is fixed at 140 mM, $A_d = \pi r^2$ is the disk patch area, $r$ is the cell radius, $\sum I_{Na}^{disk}$ is the sum of $I_{Na}$ from the disk membrane patches on the two sides of cleft, $V_{cleft} = \theta_{cleft} \cdot w \cdot \pi r^2$ is the cleft volume, and $\tau_{Na}^{cleft}$ is the time constant for cleft refilling. Parameter $\theta_{cleft}$ accounts for the cleft volume fraction that comprises the Na$^+$ nanodomains localized at the intercalated disk.

The LRd model accounts for dynamic changes in the ionic concentrations of intracellular Na$^+$, K$^+$, and Ca$^{2+}$, and the HRd model, in addition to these three, also accounts for intracellular Cl$^-$, in the axial and disk compartments. The dynamics of the intracellular concentration of ion X in compartment $j$ are governed by

$$\frac{d{[X]_j}}{dt} = -\frac{A_{tot}^{X}}{z F V_j} + \frac{[X]_j^{-1} - [X]_j}{\tau_X} + \frac{[X]_j^{+1} - [X]_j}{\tau_X},$$
where \( A_j \) is the compartment surface area, \( I_{X, \text{tot}}^j \) is the total sum of currents carried by ion \( X \), \( V_j \) is the volume associated with compartment \( j \) (\( V_j = L_p \cdot \pi r^2 \) for axial compartment, and \( V_j = L_p \cdot \pi r^2/2 \) for disk compartment), \( L_p \) is the patch length, and \( \tau_X \) is the time constant for transfer between compartments. No intercellular ionic flux were assumed. Time constants are given by \( \tau_X = L_p^2 / (2D_X) \), where diffusion coefficients (in units of \( \mu m^2 ms^{-1} \)) are \( D_{Na} = 0.6^{15}, D_K = 1.3^{16}, D_{Ca} = 0.25^{17}, \) and \( D_{Cl} = 0.6 \) (estimated to be equal to \( Na^+ \)), such that (in units of ms) \( \tau_{Na} = 83.3, \tau_K = 38.5, \tau_{Ca} = 200, \) and \( \tau_{Cl} = 83.3 \).

A subspace compartment was diffusively coupled to each intracellular compartment, containing \( Na^+ \) and \( Ca^{2+} \). \( Ca^{2+} \) influx via L-type calcium channels and 20% of \( Na^+\)-\( Ca^{2+} \) exchange (NCX) occurs in the subspace compartments. We assumed that NCX and \( Na^+\)-\( K^+ \) ATPase (NaK) activity is uniformly distributed between the axial compartments and not present at the disks. The equations governing the dynamic of subspace \( [Na^+] \) and \( [Ca^{2+}] \) in network and junctional sarcoplasmic reticulum, NSR and JSR, respectively, included transfer flux terms between adjacent subspace compartments, with time constant given by \( \tau_{Ca} \). The equations governing subspace \( [Na^+] \) and \( [Ca^{2+}] \) also included transfer flux between the subspace and the corresponding intracellular compartment, with time constant of 0.2 ms\(^{12} \).

To match experimental measurements for CV and EAD formation, the maximum conductances for \( I_{Na} \) and \( I_{CaL} \) were tuned for the different combinations of species and sodium channel mutation, given in Table S2. In the LRd model, to facilitate EADs, the L-type calcium current steady-state activation curve was shifted -2 mV. To maintain calcium homeostasis, JSR release half-saturation constant \( K_r^{rel} \) was increased from 1 to 1.5 mM, and the release time constant was increased by 50%.

**Numerical integration**

We applied a numerical integration scheme similar to Kucera et al.\(^{10} \). At each time point \( t \), we calculated the membrane current for each patch, based on the membrane patch potentials \( V_m \) and gating variable values. By applying Kirchhoff’s current law at every node and assuming that the membrane currents do not change over a small time interval \( \Delta t_1 = 0.5 \mu s \), i.e., between time \( t \) and \( t+\Delta t_1 \), we obtained a system of coupled first-order differential equations and algebraic relationships. We numerically integrated this system using a forward Euler method, with time step \( \Delta t_2 = \Delta t_1/10 = 0.05 \mu s \), and solved for all intracellular and extracellular potentials until time \( t+\Delta t_1 \). Gating variables and ionic concentrations were numerically integrated between time \( t \) and \( t+\Delta t_1 \) using a forward Euler method, with time step \( \Delta t_1 \). Using values for the membrane patch potentials \( V_m \) and gating variables, membrane currents were then recomputed at time \( t+\Delta t_1 \).

Initial conditions for each cell in the myocyte strand were established by simulating the single cell for 100 beats at a given pacing rate. Propagating electrical waves were initiated by applying a 0.5-ms stimulus current of amplitude -800 \( \mu A/cm^2 \) in the two center axial nodes (i.e., nodes 5 and 6) of cells 1 to 5. Myocyte strands were first paced to a steady-state (or a steady-state pattern for the case of the EAD-no EAD alternation
observed for mutant myocytes strands at long cycle lengths and wide cleft widths), and then simulations were run for an additional 10 beats for APD and CV measurements.

CV was computed by linear regression of the activation times (defined as the time when $V_m$ crosses above -60 mV) of cells 15 to 35. APD was computed by the difference of the repolarization times (defined as the time when $V_m$ crosses below -60 mV) and activation times in the center node of cells 15 to 35. To account for beat-to-beat variation, the maximum APD and CV values over the final 4 beats, after pacing to steady-state, are measured and reported.

**Expanded Results**

Propagation in wild-type and mutant strands

Figure S1 shows space-time plots of the transmembrane potential ($V_m$) during one beat for the four simulations shown in Figure 3. The left panel shows the first 30 ms following stimulation of the first five cells and demonstrates that, in both wild-type (WT) and mutant strands, CV is reduced as cleft width $w$ increases. As shown in Figure 3, in mutant (Y1795C) strands, EADs were masked for narrow cleft width ($w = 10$ nm), and unmasked for wide cleft width ($w = 50$ nm).

Beat-to-beat alternation in EADs in mutant myocytes

We ran single cell simulations of the mutant myocyte to determine the mechanism for the beat-to-beat EAD-no EAD alternation observed at slow cycle lengths (Figure S2A). We found that on the beat in which an EAD is triggered (red), the probability of the Na$^+$ channel being in the lower open (LO) state (see Figure 1C), the LO state associated with burst mode gating, was slightly larger following the AP upstroke, compared with the beat in which an EAD was not triggered (black). This led to a slightly larger amplitude late $I_{Na}$ that triggered the EAD. In Figure S2B, we clamped the $I_{Na}$ current to the time course for the beat in which an EAD was (red) or was not (black) present, and found that this led to EADs either present or not present, respectively, on every beat. We also clamped the time course for the Na$^+$ channel gating variables, i.e., the 13 states in the Na$^+$ channel Markov chain model, and found that this similarly led to EADs either present or not present on every beat (Figure S2C).

EAD suppression does not depend on gap junctional coupling

In order to match the CV decrease previously observed in experiments as cleft width increases$^{1,6}$, the gap junctional resistance value was increased from the baseline value in the Kucera et al.$^{10}$ EpC modeling. However, importantly, we found that the gap junctional resistance did not influence EAD suppression. In Figure S3A, we plot the transmembrane potential ($V_m^{disk}$), sodium channel open probability ($p_{open,Na}^{disk}$), sodium current ($I_{Na}^{disk}$), and sodium reversal potential ($E_{Na}^{disk}$) for different gap junctional resistance values ($R_{gap}$), both larger (blue) and smaller (green, magenta, black) the value (red) used in our study. Critically, the negative feedback mechanism underlying EAD suppression is present in the simulations, for all values of $R_{gap}$. As $R_{gap}$ decreases, the
peak $I_{Na}$ is slightly smaller, such that the cleft Na$^+$ ion depletion during the action potential stroke is slightly reduced, which in turn results in a slightly larger $E_{Na}$ following the upstroke. As a consequence, APD is slightly larger at some cleft width values for reduced $R_{gap}$ (Figure S3B). Nonetheless, EAD suppression and small APD values observed for cleft widths less than 20 nm is observed for all gap junctional resistances.

As expected, increasing $R_{gap}$ reduces CV at all cleft widths (Figure S3C). Consistent with prior computational studies\textsuperscript{10, 18, 19}, including the work by Kucera et al.\textsuperscript{10}, we found that for smaller values of $R_{gap}$, CV increases monotonically as cleft width increases, while for larger values of $R_{gap}$, is a biphasic function of cleft width, illustrated in the plot of CV normalized to its maximum value over the 5-50 nm cleft range (Figure S3D). The baseline $R_{gap}$ value for this study was chosen to match the decrease in the CV observed by the Poelzing lab experimentally over the approximately 10-25 nm cleft width range, with the closest match to absolute CV values\textsuperscript{1, 6}.

**Sodium channel localization at the intercalated disk**

We found that high Na$^+$ channel localization at the ID is required to mask EADs in narrow cleft mutant strands (Figure S4). EADs were masked when 90\% (blue) and 70\% (green) of Na$^+$ channel were localized at the ID (blue). However, EADs were unmasked when localization was reduced to 50\% (red) or 10\% (black).

However, unlike the case where increased late $I_{Na}^{disk}$ was responsible for triggering EADs, as demonstrated in Figure 3, removing Na$^+$ channels from the cleft reduced late $I_{Na}^{disk}$, due to reduced total conductance in the ID membrane patch. As a result, reducing Na$^+$ channel localization in the disk elevated $P_{Na}^{disk}$, reducing the negative feedback mechanism that decreases the late $I_{Na}^{disk}$, producing a smaller but more persistent late $I_{Na}^{disk}$. Furthermore, Na$^+$ channels now localized to the lateral membrane were exposed to an invariant extracellular [Na$^+$]$_e$ ([Na$^+$]$_e^{bulk}$), resulting in no negative feedback on $I_{Na}$ and producing a persistent lateral late $I_{Na}$. These two mechanisms in combination prolonged APD, promoted EADs, and created a highly arrhythmogenic substrate for narrow cleft width strands.

The relationship between APD and cleft width can be found in Figure S4B for four different Nav1.5 disk localization levels. With a high density of Nav1.5 in the disk (90\%, blue) increasing cleft width increased APD and promoted the formation of EADs. As Nav1.5 localization was reduced to 70\% (green) and 50\% (red), APD prolongation and EAD formation occurred at narrower cleft widths. Finally, reducing Nav1.5 localization to 10\% (black) removed APD and EAD dependence on cleft width, such that EADs were unmasked for all cleft widths.

The relationship between CV, Nav1.5 localization, and cleft width followed a similar pattern as APD prolongation and EAD formation. Specifically, the biphasic CV dependence on cleft width was maintained with high Nav1.5 disk localization (Figure S4C, 90\% and 70\%, blue and green, respectively). CV moderately increased with cleft
width when Nav1.5 localization was reduced to 50% (red), and CV was essentially independent of cleft width with 10% Nav1.5 localization (black).

**EAD suppression via Na\(^+\) depletion in a non-junctional restricted extracellular space**

Simulation results in Figure 6 show that [Na\(^+\)]\(_{\text{cleft}}\) depletion is critical to the ionic self-attenuation mechanism that reduced the late \(I_{\text{Na}}\) and masked EADs, and the results shown in Figure S4 demonstrate that Nav1.5 localization is also necessary for EAD suppression. Here, we determine whether EAD masking specifically requires Na\(^+\) ion depletion from a junctional extracellular cleft, or whether this phenomenon occurs due to depletion of any restricted extracellular space. To investigate this, we consider a simpler model, in which each myocyte \(i\) is represented by a single membrane patch and electrical conduction is represented by the standard discretized cable equation,

\[
\frac{d V_m(i)}{dt} + I_{\text{ion}}(i) = \frac{V_m(i+1) - V_m(i)}{R_{\text{gap}}} + \frac{V_m(i-1) - V_m(i)}{R_{\text{gap}}},
\]

and the extracellular sodium concentration in an axial restricted extracellular space is governed by

\[
\frac{d [\text{Na}^+]_e(i)}{dt} = -A_e \sum_{\text{Na}} [\text{Na}^+]_e(i) \tau_{\text{Na}}^{-1} + \frac{[\text{Na}^+]_{\text{bulk}} - [\text{Na}^+]_e(i)}{\tau_{\text{Na}}^{-1} \tau_{\text{Na}}^{-1} [\text{Na}^+]_e(i)} + \frac{[\text{Na}^+]_{\text{cleft}} - [\text{Na}^+]_e(i)}{\tau_{\text{Na}}^{-1} \tau_{\text{Na}}^{-1} [\text{Na}^+]_e(i)},
\]

where \(V_e\) is the extracellular space volume, \(A_e\) is the membrane patch surface area (described in more detail below), and \(\tau_{\text{Na}}^{-1} = L^2/(2D_{\text{Na}}) \approx 8.3\) sec is the time constant for diffusion between neighboring axial restricted extracellular spaces. We include the last two terms represent diffusion between neighboring axial extracellular spaces for completeness, although in practice due to the long time constant, their inclusion in the formation negligibly influences simulation results. Extracellular volume \(V_e\) was investigated for the same values as \(V_{\text{cleft}}\), corresponding to cleft width values ranging from 5 to 50 nm. We consider two cases for the membrane patch surface area \(A_e\), \(A_e = 2\pi r L\), the surface area of the axial membrane patch, and \(A_e = 2\pi r^2\), the surface area of the cleft disks, such that the surface area is comparable to the junctional cleft simulations.

We find that, as in the case for the junctional cleft extracellular space (black), EADs are suppressed in a mutant myocyte strand with non-junctional axial restricted extracellular spaces (Figure S5, red, blue), and this suppression occurs via the same negative feedback mechanism. For simulations in which the axial surface area governs [Na\(^+\)]\(_e\) dynamics (blue), the larger axial surface results in larger Na\(^+\) ion depletion, compared with the extracellular cleft simulations (black), such that EADs are suppressed over the entire extracellular volume range investigated (Figure S5B). However, the larger Na\(^+\) ion depletion also reduces peak \(I_{\text{Na}}\), such that CV monotonically decreases as the extracellular volume decreases.

For a direct comparison with the junctional cleft simulations, we artificially define [Na\(^+\)]\(_e\) dynamics as governed by the cleft surface area (red), and find that EADs are still suppressed for small extracellular cleft volumes. However, Na\(^+\) ion depletion occurs to a
much smaller extent, due to the lack of high Nav1.5 localization. As such, the late $I_{Na}$ is more pronounced, and EADs occur for myocyte strands with smaller extracellular space volumes (Figure S5B, red), compared with the cleft model (black). Further, CV is essentially independent of extracellular space volume (Figure S5C, red), due to the absence of ephaptic electric field effects.

Transition rates between “background mode” and “burst mode” sodium channel states are primarily responsible for prolonged APD and EADs in the mutant channel model

In the following parameter study description, we use the transition rate notation as in Clancy et al.$^{13}$ The discrete-state Markov chain model of the sodium channel consists of 36 transitions between 13 states. Of these 36 transition rates, all are voltage-dependent, except for the 4 transitions from the upper (U) states or “background mode” to the lower (L) or “burst mode” states ($U \rightarrow L$) and the 4 transitions from lower to upper states ($L \rightarrow U$). Most (24) of the transition rates are the same in both the wild-type and Y1795C mutant channel model, while there are 12 transition rates that are different between the wild-type and Y1795C mutant model: the four $U \rightarrow L$ transitions ($UC_3 \rightarrow LC_3$, $UC_2 \rightarrow LC_2$, $UC_1 \rightarrow LC_1$, $UO \rightarrow LO$), the four $L \rightarrow U$ transitions ($LC_3 \rightarrow UC_3$, $LC_2 \rightarrow UC_2$, $LC_1 \rightarrow UC_1$, $LO \rightarrow UO$), $\alpha_2$ (governs $UO \rightarrow UIF$), $\beta_{13}$ (governs $LO \rightarrow LC_1$, $UO \rightarrow UC_1$), and $\beta_2$ (depends on $\alpha_2$ and $\beta_{13}$ via microscopic reversibility and governs $UIF \rightarrow UO$).

Specifically, $\alpha_2$ and $L \rightarrow U$ transitions are reduced in the mutant model, and $\beta_{13}$ and $U \rightarrow L$ transitions are increased in the mutant model.

In order to assess to what extent the mutant channel transition rates promote EADs in myocyte strands with wide clefts, we performed a parameter study (consisting of 31 simulations) in which we sequentially varied $\alpha_2$, $\beta_{13}$, and the $L \approx U$ rates (varied together) from their respective wild-type to mutant channels values: Simulation 1 corresponded to the wild-type parameter set. Simulations 2-11 corresponded with $\alpha_2$ linearly decreasing from wild-type to mutant value ($\beta_{13}$ and the $L \approx U$ rates were fixed at wild-type value). Simulations 12-21 corresponded with $\beta_{13}$ linearly increasing from wild-type to mutant value ($\alpha_2$ was fixed at mutant value; the $L \approx U$ rates were fixed at wild-type values). Simulations 22-31 corresponded with the $L \rightarrow U$ and $U \rightarrow L$ rates linearly decreasing and increasing, respectively, from wild-type to mutant values ($\alpha_2$ and $\beta_{13}$ were fixed at mutant value, such that simulation 31 corresponded with the mutant value parameter set). We performed additional parameter studies in which the order of the altered parameters is varied, for all of the 6 possible combinations of ordering $\alpha_2$, $\beta_{13}$, and the $L \approx U$ rates.

In Figure S6, APD is shown as a function of the wild-type to mutant parameter value transition for cleft widths of 30, 40, and 50 nm. Importantly, we find that, regardless of the order in which the transition rates were varied from wild-type to mutant values, mutant $L \approx U$ rates promoted EADs most prominently, such that the APD for mutant $L \rightarrow U$ and $U \rightarrow L$ rates and wild-type $\alpha_2$ and $\beta_{13}$ rates (blue and magenta) was longer than APD for the strictly mutant myocyte strands. Similarly, when mutant $L \approx U$ rates were the second (red and green) or third (black and cyan) transition rate varied from wild-type
to mutant values, APD dramatically increased. Interestingly, increasing $\beta_{13}$ from wild-type to mutant values either had no effect or decreased APD, while decreasing $\alpha_2$ from wild-type to mutant values also had minimal effect on APD, further emphasizing that the mutant $L \rightleftharpoons U$ rates are primarily responsible for prolonged APD and EADs. Further, these findings were consistent for myocyte strands with cleft widths of 30, 40, and 50 nm, suggesting that the transition rates primarily responsible for promoting EADs did not specifically depend on cleft width.

_masking of EADs does not depend on the specific sodium channel mutation nor species_ The transmembrane potential ($V'_m$), sodium channel open probability ($p_{\text{open,Na}}$), sodium current ($I_{\text{Na}}^{\text{disk}}$), sodium reversal potential ($E_{\text{Na}}^{\text{disk}}$), APD, and CV are shown for WT and mutant (I1768V) guinea pig myocyte strands (Figure S7A), for WT and mutant (Y1795C) canine myocyte strands (Figure S7B), and for WT and mutant (I1768V) canine myocyte strands (Figure S7C). As for the mutant (Y1795C) guinea pig myocyte strands illustrated in the main text, EADs were masked in narrow cleft and unmasked in wide cleft strands. Critically, reduced late $I_{\text{Na}}^{\text{disk}}$, due to cleft Na$^+$ depletion and reduced $E_{\text{Na}}^{\text{disk}}$, was the primary underlying mechanism for concealing EADs, independent of the mutant type or species.
Supplemental Figures and Figure Legends

Figure S1. Space-time plots of propagation in wild-type and mutant strands. The transmembrane potential ($V_m$) is shown as a function of space and time, during the final beat of 1000-ms cycle length pacing. Color maps are shown for wild-type (WT) and mutant (Y1795C) myocyte strands with narrow ($w = 10 \text{ nm}$) and wide ($w = 50 \text{ nm}$) cleft widths.
Figure S2. Beat-to-beat EAD alternation in mutant myocyte simulations. (A) Transmembrane potential ($V_m$, top), sodium current ($I_{Na}$, middle), and probability of the sodium channel lower open (LO) state ($p_{LO,Na}$, bottom) are shown as a function of time for successive beats, in which an EAD was (red) and was not (black) present. (B) $V_m$ is shown as a function of time during simulations in which $I_{Na}$ is clamped for each beat to the time course for the EAD beat (red) or no EAD beat (black), leading to EADs or no EADs on every beat. (C) $V_m$ is shown as a function of time during simulations in which the Na$^+$ channel gating variables, i.e., the 13 states in the Markov chain gating model (Figure 1C), are clamped for each beat to the time course for the EAD beat (red) or no EAD beat (black), leading to EADs or no EADs on every beat.
Figure S3. Gap junctional coupling does not influence EAD suppression. (A) Transmembrane potential ($V_{\text{disk}}^{\text{m}}$), Na$^+$ channel open probability ($p_{\text{open,Na}}^{\text{disk}}$), Na$^+$ current ($I_{\text{disk}}^{\text{Na}}$), and Na$^+$ reversal potential ($E_{\text{Na}}^{\text{disk}}$) at the upstream disk membrane patches in cells 10 and 40 are shown as a function of time, during 1000-ms cycle length pacing, for mutant myocytes in strands with variable gap junctional resistance. Cleft width $w = 10$ nm. (B) Action potential duration (APD), (C) conduction velocity (CV), and (D) CV (normalized to the maximum value over the cleft width range) are shown as a function of cleft width in mutant myocyte strands for variable gap junctional resistance.
Figure S4. Na⁺ channel localization is necessary to mask EADs. (A) Transmembrane potential ($V_{m}^{\text{disk}}$), Na⁺ channel open probability ($p_{\text{open},Na}^{\text{disk}}$), Na⁺ current ($I_{Na}^{\text{disk}}$), and Na⁺ reversal potential ($E_{Na}^{\text{disk}}$) at the upstream disk membrane patches in cells 10 and 40 are shown as a function of time, during 1000-ms cycle length pacing, for mutant myocytes with variable Na⁺ channel intercalated disk localization. Cleft width $w = 10$ nm. (B) Action potential duration (APD) and (C) conduction velocity (CV) are shown as a function of cleft width in mutant myocyte strands for variable localization.
Figure S5.  EADs are masked in an axial restricted extracellular space. (A) Transmembrane potential ($V_m$), Na$^+$ channel open probability ($p_{open,Na}$), Na$^+$ current ($I_{Na}$), and Na$^+$ reversal potential ($E_{Na}$) at the upstream membrane patches in cells 10 and 40 are shown as a function of time, during 1000-ms cycle length pacing, for mutant myocytes with either a cleft (black) or axial restricted extracellular space, in which surface area (SA) is given by the axial (red) or cleft (blue) surface area (see text for details). Cleft volume $w = 0.38 \, \mu m^3$, correspond to cleft width of 10 nm. (B) Action potential duration (APD) and (C) conduction velocity (CV) are shown as a function of cleft volume in mutant myocyte strands.
Figure S6. The mutant channel transition rates between “background” and “burst” mode gating prolong APD and promote EADs. APD is shown as a function of the parameter transition from wild-type (WT) to mutant (Y1795C) for cleft width values of 30 (left), 40 (middle), and 50 nm (right). The x-axis tick marks 1-4 correspond with simulation 1 (WT), 11, 21, and 31 (Y1795C), respectively, as described in the supporting text.
Figure S7. Masking of EADs does not depend on the specific sodium channel mutation nor species. (Left) The transmembrane potential ($V_m$), sodium channel open probability ($P_{open,Na}^{disk}$), sodium current ($I_{Na}^{disk}$), and sodium reversal potential ($E_{Na}^{disk}$) are shown as a function of time for (A) wild-type (WT) and mutant (I1768V) guinea pig myocyte strands; (B) WT and mutant (Y1795C) canine myocyte strands; and (C) WT and mutant (I1768V) canine myocyte strands. (Right, top) Action potential duration (APD) and (bottom) conduction velocity (CV) are shown for WT and mutant myocyte strands as a function of cleft width $w$. 
### Supplemental Tables

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<tr>
<td>$r$</td>
<td>Cell radius</td>
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<td>Extracellular resistence</td>
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<td>Intercellular cleft width</td>
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<td>Radial cleft resistance</td>
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<td>Varied (depends on $w$)</td>
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**Table S1.** Electrical parameters and equations from Kucera et al.\textsuperscript{10} ephaptic coupling model. \* $R_{gap}$ was adjusted from the Kucera et al. model to fit the dependence of CV on intercellular cleft width $w$ measured in experiments\textsuperscript{1,6}. See Figure S3 and supporting results for the dependence of APD and CV on $R_{gap}$. 


<table>
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**Table S2.** Scaling factors and cleft parameters for myocyte ionic models and Na$^+$ channel mutations.
## Table S3.

Data set and statistical comparisons for Figure 2B. APD measurements are given as a function of cycle length, and the p-value for the statistical comparison relative to control and ATXII are shown. P-values less than 0.050 are shown in bold.

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<th>St. Dev (ms)</th>
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Table S4. Analysis of the APD-cycle length relationship using a linear mixed effects statistical model to compare curve intercept and slope.
Supplemental References


