Genesis of Phase-3 Early Afterdepolarizations and Triggered Activity in Acquired Long QT Syndrome

Running title: Maruyama et al.; Mechanism of phase-3 EAD

Mitsunori Maruyama, MD, PhD¹; Shien-Fong Lin, PhD¹; Yuanfang Xie, PhD²; Su-Kiat Chua, MD¹; Boyoung Joung, MD, PhD¹; Seongwook Han, MD, PhD¹; Tetsuji Shinohara, MD, PhD¹;

Mark J. Shen, MD¹; Zhilin Qu, PhD²; James N. Weiss, MD²; Peng-Sheng Chen, MD¹

¹Krannert Institute of Cardiology and the Division of Cardiology, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN; ²Cardiovascular Research Laboratory, Departments of Medicine (Cardiology) and Physiology, David Geffen School of Medicine, University of California, Los Angeles, CA.

Corresponding Author:

Peng-Sheng Chen, MD,
1800N. Capitol Ave, E475,
Indianapolis, IN 46202.
Phone: 317-962-0145
Fax: 317-962-0588
E-mail: chenpp@iupui.edu

Journal Subject Codes: [132] Arrhythmias-basic studies
Abstract:

**Background**—Both phase-2 and phase-3 early afterdepolarizations (EADs) occur in long QT syndromes, but their respective roles in generating arrhythmias in intact cardiac tissue are incompletely understood.

**Methods and Results**—Intracellular Ca (Ca_i) and membrane voltage (V_m) were optically mapped in a quasi 2-dimensional model of cryoablated Langendorff-perfused rabbit ventricles (n = 16). E-4031 (an I_{Kr} blocker) combined with reduced extracellular K ([K^+]_o) and Mg ([Mg^{2+}]_o) prolonged action potential duration (APD) heterogeneously and induced phase-2 and phase-3 EADs. While phase-2 EADs were Ca_i-dependent, phase-3 EADs were not. The origins of 47 triggered activity (TA) episodes were attributed to phase-2 EADs in 12 episodes (26%) and phase-3 EADs in 35 episodes (74%). When phase-2 EADs accompanied phase-3 EADs, they accentuated APD heterogeneity, creating a large V_m gradient across the boundary between long and short APD regions from which TA emerged. The amplitude of phase-3 EADs correlated with the V_m gradient (r = 0.898, P < 0.001). Computer simulation studies showed that coupling of cells with heterogeneous repolarization could extrinsically generate phase-3 EADs via electrotonic current flow. Alternatively, reduced I_{K1} caused by low [K^+]_o could generate intrinsic phase-3 EADs capable of inducing TA at the boundary zone.

**Conclusions**—Phase-3 EADs can be extrinsic due to electrotonic current across steep repolarization gradients, or intrinsic due to low I_{K1}, and do not require spontaneous sarcoplasmic reticulum Ca release. Reduction of I_{K1} by low [K^+]_o strongly promotes ventricular arrhythmias mediated by phase-3 EADs in acquired long QT syndrome due to I_{Kr} blockade.

**Key words:** action potentials; calcium; depolarization; long-QT syndrome; torsade de pointe
Introduction

Early afterdepolarizations (EADs) are strongly associated with ventricular arrhythmias in long QT syndromes (LQTS). Both phase-2 and phase-3 EADs have been described in LQTS. It is generally accepted that phase-2 EADs result from the reactivation of $I_{Ca,L}$ and/or spontaneous Ca release from the sarcoplasmic reticulum (SR).$^{1-3}$ The ionic mechanism of phase-3 EADs, however, is less clear. Whereas phase-2 EADs can be readily recorded from isolated myocytes as well as intact tissue, phase-3 EADs have usually been reported in intact tissue preparations, such as Purkinje fibers or ventricular muscle.$^{4-8}$ It has been suggested that spontaneous SR Ca release may underlie phase-3 EADs, since they may occur concurrently with delayed afterdepolarizations (DADs),$^{4-5}$ are facilitated by intracellular Ca (Ca$_i$) loading, and are suppressed by inhibition of Na$^+$/Ca$^{2+}$ exchanger current ($I_{NCX}$).$^{1,5}$ Alternatively, it is possible that most phase-3 EADs observed in tissue not a genuine cellular level phenomenon, but instead are a consequence of “prolonged repolarization-dependent reexcitation.” Brugada and Wellens$^9$ conjectured that if dispersion of repolarization is enhanced in LQTS, a voltage gradient between long and short APD regions could create a “boundary” current which electrotonically depolarizes the short APD region as it tries to repolarize, generating triggered activity (TA) as long as a large voltage gradient is maintained. This form of TA arising from the boundary zone was observed in a partition chamber which artificially created heterogeneous repolarization,$^{10}$ but it has not been demonstrated in the setting of LQTS in intact hearts. We tested this hypothesis by performing high-resolution optical mapping of $Ca_i$ and membrane voltage ($V_m$) in a rabbit model of acquired LQTS, accompanied by computer simulations which reproduced the experimental observations. Our combined experimental and modeling findings suggest that electrotonic interactions between a long APD region, with or without phase-2 EADs, and its
neighboring repolarizing region, is a major cause of phase-3 EADs and TA in this model. There
was no evidence that phase-3 EADs were dependent on changes in Ca, suggesting that they are
more likely to result from electrotonic interactions across boundaries with steep APD gradients,
rather than local spontaneous SR Ca release.

Methods
2-dimensional Epicardial Layer of Langendorff-perfused Rabbit Ventricles

This study protocol was approved by the Institutional Animal Care and Use Committee
of Indiana University School of Medicine, and conforms to the guidelines of the American
Heart Association. New Zealand White female adult rabbits (n = 16) were anesthetized with
sodium pentobarbital (50 mg/kg). The heart was rapidly excised and perfused at 25 to 30
mL/min using Langendorff-perfusion system with oxygenated Tyrode’s solution (in mmol/L:
NaCl 125, KCl 4.5, NaHCO3 24, NaH2PO4 1.8, CaCl2 1.8, MgCl2 0.5, and glucose 5.5) with a
pH of 7.40 ± 0.05. We performed cryoablation using a 7-cm SurgiFrost probe (CryoCath
Technologies Inc, Quebec, Canada) with the probe temperature of -135°C for 3 minutes for the
right ventricle (RV) and 5 minutes for the left ventricle (LV). During the cryoablation, the
epicardium was protected by immersing the heart into warm (37°C) Tyrode’s solution. After the
study, we sectioned the heart horizontally to confirm the surviving epicardial layer (<1 mm)
with 1% triphenyltetrazolium chloride (Online Supplement Figure I).

Experiment Protocol

Simultaneous Ca, and Vm mapping was performed as described previously.11 EADs and
TAs were induced by bradycardia following endocardial cryoablation, a selective IKr blocker E-
4031 (0.5 μmol/L, Tocris Bioscience, Ellisville, MO), and 50% reduction of extracellular potassium ([K⁺]₀) and magnesium ([Mg²⁺]₀) concentrations. We also examined the effects of BAPTA-AM (20 μmol/L, Tocris) to determine the role of Cai in EADs (n = 4). Pseudo-ECG was recorded utilizing the chamber solution as a volume conductor. Bipolar electrodes were attached to the RV outflow tract for pacing. In 3 hearts, transmembrane potentials (TMPs) were recorded during optical mapping with a standard glass microelectrode, as described previously. The heart was immersed in the heated tissue chamber (37°C) throughout the study to avoid a temperature gradient of the mapped surface. Contraction was inhibited with 10 μmol/L blebbistatin (Tocris) during optical mapping.

**Definitions**

*Cai re-elevation*: Increase in Cai during the plateau of action potential (AP) following the first upstroke of Cai transient.

*Phase-2 EAD*: Depolarizing afterpotentials occurring at the plateau level of AP. Depolarization during the plateau phase was not considered as phase-2 EAD if the depolarization was induced by electrotonic interaction of an activation wavefronts passing near the pixel of interest.

*Phase-3 EAD*: Afterpotentials which retard the expected course of phase-3 AP repolarization. Previous studies consistently defined phase-3 EADs by this criterion, even though no actual depolarization is observed.

*TA*: Propagated responses evoked by EADs that inscribe a QRS complex on the pseudo-ECG.

*Torsades de Pointes (TdP)*: Polymorphic VT characterized by undulating changes in the QRS axis on pseudo-ECG.
Data Analysis

The methods for optical data processing were reported elsewhere. We defined the amplitude of baseline $V_m$ and $Cai$ transient as 1 arbitrary unit (AU). The $V_m$ phase map was made using a time-delay embedding method. To construct $V_m$ gradient (VG) map, the VG at the pixel with coordinates $(n, m)$ was calculated with the larger of the absolute value of $(V_m(n-3, m) - V_m(n+3, m))/\text{distance}$ and $(V_m(n,m-3) - V_m(n, m+3))/\text{distance}$. Because the inter-pixel distance was 0.35 mm, the pixel distance used in the gradient calculation over 6 pixels was 2.1 mm. APD was measured at 70% repolarization. APD dispersion was defined as a time difference between maximal and minimal APDs in the mapped area. The amplitude of phase-3 EAD was measured as the difference between the diastolic resting $V_m$ and the first deviation from smooth contour during phase-3 repolarization.

Continuous variables were expressed as mean ± SEM unless otherwise indicated. Statistical analysis was performed by paired t-test to compare VG values with and without TAs, and by one-way repeated measure ANOVA to compare the data for different pacing cycle lengths (PCLs). The relationship between maximal VG and APD dispersion, and between phase-3 EAD amplitude and VG were tested with Pearson’s correlation. $P \leq 0.05$ was considered statistically significant.

Computer simulation

Simulations corresponding to isolated myocytes and one-dimensional (1D) cables were performed. AP models in the simulations were modified from the rabbit ventricular action potential model developed by our group. To model the effects of E-4031 and hypokalemia, $I_{Kr}$ was blocked and the extracellular potassium concentration $[K^+]_0$ was reduced to 2.7 mmol/L.
The details of the AP models are shown in Online Supplemental Materials. For the isolated myocytes, the governing differential equation for voltage is:

\[
C_m \frac{dV}{dt} = -(I_{ion} + I_{stim}) \tag{1}
\]

where \(C_m\) is the membrane capacitance set at 1 \(\mu\)F/cm\(^2\), \(I_{ion}\) is the total membrane ionic currents, and \(I_{stim}\) is the stimulation current, a square pulse of strength 40 \(\mu\)A/cm\(^2\) and duration 1 ms. The differential equation for voltage in the 1D cable is:

\[
\frac{\partial V}{\partial t} = -I_{ion} / C_m + D \frac{\partial^2 V}{\partial x^2} \tag{2}
\]

where \(D\) is the diffusion coefficient with the value of 0.0002 cm\(^2\)/ms. No-flux boundary conditions were used. Numerically, Eq.2 was discretized with \(\Delta x = \Delta y = 0.015\) cm and integrated with a forward Euler method with an adaptive time step varying from 0.01 ms to 0.1 ms.

**Results**

**Phase-2 EADs and Heterogeneous Repolarization**

Exposure to 0.5 \(\mu\)mol/L E-4031 in combination with a 50% reduction in \([K^+]_o\) and \([Mg^{2+}]_o\) prolonged QT intervals and induced R-on-T ectopic beats in all hearts studied (n = 16). In 6 of 16 hearts, data were also collected before and shortly (approximately 5 min) after lowering \([K^+]_o\) and \([Mg^{2+}]_o\) to measure QT interval and APD during stable pacing. R-on T ectopic beats usually appeared 5-15 min after reducing \([K^+]_o\) and \([Mg^{2+}]_o\). Because these ectopic beats caused irregular cycle lengths, QT and APD were not measured when there were ectopic activities. Changes in QT interval and APD are summarized in Table 1. As the QT interval was prolonged, both APD dispersion and maximal VG during repolarization increased. The maximal
VG correlated with APD dispersion (Online Supplement Figure II). APD prolongation was spatially heterogeneous, causing “island”-like long APD regions to emerge (Figure 1A), with a large VG at the boundary zone between the long and short APD regions. The size of the long APD region was dependent on cycle length, but independent of activation sequence (Online Supplement Figure IIIA). The spatial distribution of long APD regions varied among individual hearts, although gradual changes in the distribution occurred over time in the same heart (Online Supplement Figure IIIB). Phase-2 EADs and R-on-T ventricular ectopic beats were observed after reducing \([K^+]_o\) and \([Mg^{2+}]_o\). Phase-2 EADs further enhanced heterogeneity of repolarization, since the phase-2 EADs occurred exclusively in long APD regions.

**Phase-3 EADs**

Phase-3 EADs became manifest after E-4031 infusion with low \([K^+]_o\) and \([Mg^{2+}]_o\) (Figure 1B). The cycle-length dependence of phase-3 EADs was comparable to previously reported observations (Online Supplement Figure IV).\(^8\)\(^,\)\(^15\) Phase-3 EADs were also confirmed in TMP recordings (Online Supplement Figure V). No transition from phase-2 EADs to phase-3 EADs was observed (Online Supplement Figure VI). Simultaneous recording of \(V_m\) and \(C_{ai}\) during phase-2 and phase-3 EADs (Figure 2A) revealed that the onset of phase-2 EADs did not precede that of \(C_{ai}\) re-elevation at the site of phase-2 EAD origin, as reported previously.\(^3\) Of 55 episodes of phase-2 EAD arising from the mapped area, \(C_{ai}\) re-elevations occurred earlier than phase-2 EADs by \(26 \pm 2\) ms in 44 episodes (80%), or synchronously in 11 episodes (20%). In contrast, changes in \(V_m\) always preceded changes in \(C_{ai}\) during phase-3 EADs by \(25 \pm 3\) ms, suggesting that spontaneous SR Ca release may underlie the development of phase-2 EADs, but not for phase-3 EADs. To further confirm the role of \(C_{ai}\) in EADs, we tested the effect of the
Ca, chelator BAPTA-AM (20 μmol/L, n = 4). The maximal amplitude of Ca, transient was decreased by 69 ± 4% after 60 min of BAPTA-AM infusion. BAPTA-AM abolished phase-2 EADs, but phase-3 EADs persisted after BAPTA-AM loading in all hearts studied (Figure 2B). These findings suggest that whereas phase-2 EADs are Ca, dependent, phase-3 EADs are not. Interestingly, the largest phase-3 EADs always occurred at the boundary between long and short APD regions (Figure 3A). At the site with the largest phase-3 EAD, the EAD amplitude correlated with the VG at the time of the EAD onset (\(r = 0.898, P < 0.001\), Figure 3B). Taken together, the findings suggest that electrotonic currents flowing from more positive \(V_m\) in long APD regions to shorter APD regions can cause phase-3 EADs at the boundary zone, without any requirement for SR Ca release.

**EAD-Mediated TAs**

No TAs occurred with E-4031 alone, but 114 spontaneous episodes of TAs were observed after a 50% reduction in \([K^+]_o\) and \([Mg^{2+}]_o\). Forty-seven TA episodes (41%) originated from the mapped area, with 3 modes of initiation identified, as described below.

First, TAs could be directly induced by phase-2 EADs. Figure 4 shows an example. During the early phase of repolarization, \(V_m\) and Ca, levels increased again (i.e. phase-2 EAD and Ca, re-elevation, respectively) near the center of the long APD region (site (a), squares in \(V_m/\text{Ca},\) ratio maps). The phase-2 EAD spread within the long APD region, but did not directly propagate beyond. However, TA emerged from the boundary between long and short APD regions where excitability had recovered (site (c), yellow arrowhead). TA rapidly propagated through the more repolarized area (i.e. short APD region), which inscribed the QRS complex on
pseudo-ECG. The second hump (asterisks) was generated in long APD region as the TA wavefront traveled around the long APD region, suggesting an electrotonic depolarization.

Second, TAs could arise directly from phase-3 EADs in the absence of phase-2 EADs. Figure 5 shows an example of TA mediated by a phase-3 EAD. APD in the mid-LV epicardium was heterogeneously prolonged by E-4031 with low $[K^+]_o$ and $[Mg^{2+}]_o$. There were no phase-2 EADs in long APD region (site (a)). TA then emerged 366 ms after ventricular pacing at a PCL of 2000 ms (yellow arrowhead). Note that the earliest activation did not reside in long APD region (site (a)), but rather occurred at the inferior boundary between long and short APD regions (site (b)). Phase maps revealed that the course of repolarization (in yellow to red) at the inferior boundary suddenly reversed, generating a phase-3 EAD which induced TA. The VG across the boundary was the maximal at that time.

Third, phase-3 EAD-mediated TAs could also occur in association with phase-2 EADs. In Figure 6, a phase-2 EAD and Ca$^+$ re-elevation occurred (squares in $V_m$/Ca$^+$ ratio maps) from long APD region located at the basal LV and RV. Afterwards, TA emerged from the boundary zone (yellow arrowhead). However, phase-2 EADs (black filled circle) failed to cause TA, and a phase-3 EAD (black unfilled circle) was present at the earliest activation site of the TA (site (b)). The phase-3 EAD also coincided with a high VG, but in this case, phase-2 EAD aggravated the VG by further delaying repolarization in the long APD region (site (a)). Therefore, interaction between phase-2 and phase-3 EADs was important for this mode of TA initiation.

Of 47 episodes of TA, phase-2 EADs directly induced TA in 12 episodes (26%), while phase-3 EADs induced TA in 35 episodes (74%). The majority (63%) of the latter phase-3 EAD-mediated TA episodes were associated with electrotonic interaction with phase-2 EADs. The VG at the origin of the phase-3 EAD-mediated TA was the maximal or submaximal within
the mapped area (average 94 ± 1% [range 71% to 100%] of the largest VG during repolarization). In 10 hearts in which the site of the TA origin was mapped and the maximal VG without TAs was measured during E-4031 infusion with reduced [K⁺]₀ and [Mg²⁺]₀, the VG at the TA origin was significantly higher than the maximal VG in the absence of TA (0.40 ± 0.03 AU/mm versus 0.34 ± 0.03 AU/mm, \( P = 0.016 \)).

**Mechanisms of VT**

Thirty-eight episodes of VT were observed, including 8 monomorphic VTs, 25 polymorphic VTs, and 5 VTs with TdP-like morphologic features. The mechanism of VT was determined in 18 episodes of VT originating from the mapped area (2 monomorphic VTs, 13 polymorphic VTs, 3 TdPs). Repetitive phase-3 EAD-mediated TA arising from the same site at the boundary was responsible for all monomorphic VTs (Figure 7A, Movie I). Note that the long APD region never fully repolarized during VT (asterisks) probably due to electrotonic depolarization by TA (blue arrows), whereby the high VG was maintained. Two mechanisms accounted for polymorphic VT. Repetitive focal activations from single or multiple foci shifting from beat to beat caused 11 episodes (Figure 7B, Movie II). In the remaining 2 episodes, a combination of focal activations and macro-reentry revolving around long APD region was responsible (Figure 7C, Movie III). Three episodes of TdP were induced by phase-3 EAD-mediated TA associated with phase-2 EADs (Online Supplement Figure VII), which initiated a reentrant rotor with a drifting core. We did not observe TdP maintained by a focal activation mechanism.

**Computer Simulations**
To explore the role of electrotonic coupling in the genesis of phase-3 EADs and TAs in tissue with heterogeneous APD due to reduced repolarization reserve, we modified a rabbit ventricular AP model to exhibit two (for simplicity) types of AP morphology (Figure 8). When we coupled cells with short AP and prolonged AP with small phase-2 EADs, phase-3 EADs emerged at the boundary zone between these two types of cells in a 1D cable, resembling our experimental results as well as those in previous reports.4-8 Even with very large differences in APD, TA did not occur if [K+]o was normal. When [K+]o was reduced from 5.4 mmol/L to 2.7 mmol/L, delayed repolarization mimicking phase-3 EADs appeared due to the resulting reduction in \( I_{K1} \). The mechanism of low [K+]o inducing phase-3 EADs was detailed in a simulation study by Luo and Rudy.16 Reduction of \( I_{Kr} \) further potentiated the formation of phase-3 EADs due to its effect on reducing repolarization reserve. However, these phase-3 EADs also did not cause TA in uncoupled cells. When the cells were coupled under low [K+]o conditions, TA arose from the short APD side of the boundary zone, despite a smaller APD difference between two types of cells. In line with the optical mapping results, no spontaneous Ca release was present during phase-3 EADs in the AP models.

Discussion

Phase-2 EADs and TA

The ability of an EAD to propagate is favored by a more negative EAD take-off potential, allowing greater recovery of the \( I_{Ca,L} \) to facilitate propagation. This is supported by the experimental observations of Damiano and Rosen,8 who reported that phase-3 EADs, but not phase-2 EADs, induced TA. On the other hand, Yan et al17 demonstrated that phase-2 EADs were directly associated with TA and TdP in a canine LV-wedge preparation. However, the
main vector of TA activation in their study was not from the phase-2 EAD site, raising uncertainty as to how phase-2 EADs elicited propagated responses. We found that when phase-2 EADs were confined to the center of the long APD region, they did not induce TA. When phase-2 EADs occurred near or were transmitted to the boundary zone, such that they encountered partially repolarized tissue, however, a new AP could be triggered and propagate into the short APD region (Figure 4). Therefore, our results suggest that heterogeneous repolarization is necessary for phase-2 EADs to induce TA. This agrees with the finding that increased dispersion of repolarization facilitates the ability of phase-2 EADs to generate TA.17

**Mechanism of Phase-3 EADs**

Previous studies suggested elevated Cai causing enhanced \( I_{NCX} \) might underlie phase-3 EADs.\(^1,5\) However, we observed neither persistent Cai elevation nor spontaneous Cai elevation during the phase-3 EADs in the present study. It has been shown that phase-3 EADs can also occur due to \( I_{NCX} \) activation by a large Cai transient that outlasts the end of an AP.\(^18,19\) However, this type of “late” phase-3 EAD occurs when APD is shortened rather than prolonged. While \( I_{NCX} \) inhibition may suppress phase-3 EADs that occur during APD prolongation,\(^5\) the same intervention also attenuates phase-2 EADs,\(^20\) which diminishes the VG between long and short APD regions. The latter effects may suppress the phase-3 EAD indirectly. Therefore, suppression of phase-3 EAD by \( I_{NCX} \) inhibition does not necessarily indicate that phase-3 EAD is purely \( I_{NCX} \) dependent.

The higher prevalence of phase-3 EADs at slower heart rates\(^7\) with low [K\(^+\)]\(_o\)\(^8\) is compatible with our findings, since these interventions augment the VG. We found that a large VG related to heterogeneous repolarization is essential for phase-3 EADs. Liu and Laurita\(^21\)
reported that the breakthrough site of TA always occurred where the local repolarization gradient was the largest in the canine wedge model of LQTS. In the present study, we can exclude breakthrough activation from the deeper layer since the cells beyond ≈1 mm from the epicardial surface were cryoablated. Thus, TA could only arise from the epicardial site where a phase switch from repolarization to depolarization first occurred. A large VG and phase-3 EAD at the “arrhythmogenic” boundary suggests electrotonic reexcitation as the most likely underlying mechanism of TA (antegrade electrotonic interaction, red arrows in Figure 7). The TA caused by electrotonically-assisted phase-3 EAD then rapidly propagates over the short APD region, which in turn extends APD electrotonically in the long APD region (retrograde electrotonic interaction, blue arrows in Figure 7). As a result of this ping-pong interaction, the long APD region is precluded from full-repolarization even at faster heart rates, and the persistent high VG generates repetitive firing of phase-3 EAD-mediated TAs. This mechanism is consistent with “prolonged repolarization-dependent reexcitation”, in which bidirectional electrotonic interaction between long and short APD regions contributes to the maintenance of VT.

Phase-3 EADs have been reported mainly in Purkinje fibers and in the in-vivo hearts with intracellular microelectrode or monophasic AP recordings. Although Ca$_i$-dependent phase-3 EADs have been reported in isolated cardiomyocytes under some experimental conditions (e.g. K$^+$-free Tyrode’s solution), the fact that the great majority of phase-3 EADs have been recorded from multicellular preparations suggests that electrotonic interactions may be important for the development of phase-3 EAD in many long QT settings. If true, this implies that a large portion of phase-3 EADs described in the literature do not arise solely from the intrinsic ionic currents of the myocyte, but rather a represent a combination of electrotonic...
currents interacting with intrinsic ionic currents. Using computer simulation studies, we
documented this scenario by showing that a phase-3 EAD can be produced by electrotonic
coupling in tissue with a large APD gradient. In addition, inhibition of $I_{K1}$ can be the intrinsic
source of phase-3 EADs, but the synergistic effect of the intrinsic and extrinsic mechanisms of
phase-3 depolarization may be essential for ventricular arrhythmogenesis in acquired LQTS.

Clinical Implications

Our results provided new insights into the mechanisms of arrhythmogenesis in drug-
duced LQTS during hypokalemia and hypomagnesemia. Increasing heart rate with temporary
pacing is a reasonable treatment for acquired LQTS because the repolarization heterogeneity is
cycle-length dependent. Treatments targeting to phase-2 EADs may also suppress phase-3
EADs, which are major causes of TA.

Study limitations

We used E-4031 with low $[K^+]_o$ and $[Mg^{2+}]_o$ as a model of acquired LQTS. Our findings
may not be applicable to other types of LQTS. We investigated only the epicardium in this study.
However, previous studies have shown that EADs originate preferentially from Purkinje
cells$^{15,22}$ and M cells.$^{24}$ It is possible that “prolonged repolarization-dependent reexcitation” may
contribute to the generation of phase-3 EADs and TAs in Purkinje-muscle junction and the
boundary between M cells and other tissues. Our computer simulations were carried out only in
1D cable. Simulating reentry-like behavior will need to use of 2D or 3D tissue models. However,
1D simulation allows us to focus on the mechanism of EADs and TAs, which are the primary
purpose of this study.
Conclusions

A number of studies have documented the importance of heterogeneous repolarization in the ventricular arrhythmogenesis in LQTS.\textsuperscript{1-3,9,14,18,22} The present study demonstrates that heterogeneous repolarization is indispensable not only for creating a functional substrate promoting reentry, but also directly participates in generating the triggers that emerge from phase-2 and phase-3 EADs. In addition, we provide the first direct experimental evidence for “prolonged repolarization-dependent reexcitation” in intact cardiac muscle tissue.

Acknowledgements: We thank Erica Foster for her secretarial assistance.

Funding Sources: This study was supported in part by National Institutes of Health grants P01 HL78931, R01 HL78932, and 71140; a Nihon Kohden/St Jude Medical electrophysiology fellowship (Dr Maruyama); a Postdoctoral Fellowship Award from American Heart Association, Western States Affiliate (Dr Xie); the Kawata and Laubisch Endowments (Dr. Weiss) an American Heart Association Established Investigator Award (Dr Lin); and a Medtronic-Zipes endowment (Dr Chen).

Conflict of Interest Disclosures: CryoCath Technologies, Inc, provided the SurgiFrost probe.

References:


Table 1. Effects of E-4031 and Low [K\(^+\)]\(_o\) and [Mg\(^{2+}\)]\(_o\) on QT Intervals, APD, APD Dispersion, and VG (n = 6)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>E-4031 0.5 μmol/L + low [K(^+)](_o)/[Mg(^{2+})](_o)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCL, ms</td>
<td>PCL, ms</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>1000</td>
</tr>
<tr>
<td>QT intervals, ms</td>
<td>258 ± 20</td>
<td>271 ± 16</td>
</tr>
<tr>
<td>Max APD, ms</td>
<td>201 ± 22</td>
<td>212 ± 19</td>
</tr>
<tr>
<td>Min APD, ms</td>
<td>186 ± 23</td>
<td>193 ± 24</td>
</tr>
<tr>
<td>APD dispersion, ms</td>
<td>15 ± 6</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>Max VG, AU/mm</td>
<td>0.13 ± 0.04</td>
<td>0.14 ± 0.04</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. PCL indicates pacing cycle length; [K\(^+\)]\(_o\) and [Mg\(^{2+}\)]\(_o\), extracellular concentration of potassium and magnesium; APD, action potential duration at 70% repolarization; VG, membrane voltage gradient. *, †These values were obtained from 4 and 2 hearts, respectively, since R-on-T ectopic beats prevented measurements of QT and APD.
Figure Legends:

**Figure 1.** Development of phase-2 and phase-3 EADs. A, The spatial distribution of APDs and VGs during repolarization is displayed in color-scaled maps. Optical APs at the maximal (filled squares) and minimal (unfilled squares) APD sites are shown with pseudo-ECG (pECG). In addition to E-4031, reducing [K\(^+\)]\(_o\) and [Mg\(^{2+}\)]\(_o\) greatly enhanced repolarization heterogeneity. Note that the appearance of phase-2 EAD (filled circle) further increased the VGs and was associated with emergence of an R-on-T ventricular ectopic beat (asterisk). APD was not measurable in a blank area of the APD map where repolarization was interrupted by the ectopic beat. B, With E-4031 alone, there was no phase-3 EAD. Phase-3 EAD (unfilled circles) and an ectopic beats (asterisk) emerged after reduction in [K\(^+\)]\(_o\) and [Mg\(^{2+}\)]\(_o\). Phase-3 EAD is discernable as the V\(_m\) difference between the resting V\(_m\) and the first deviation from the smooth contour during phase-3 repolarization.

**Figure 2.** Role of Ca\(_i\) in EADs. A, Simultaneous Ca\(_i\) and V\(_m\) recordings for phase-2 (black filled circle) and phase-3 (unfilled circle) EADs. Left panel shows the onset of Ca\(_i\) re-elevation (red arrow) preceded that of phase-2 EAD (black arrow). Right panel shows the first deviation of V\(_m\) (black arrow) was followed by the deviation of Ca\(_i\) (red arrow) from the expected trajectory. B, Effect of BAPTA-AM (20 \(\mu\)mol/L) on EADs. BAPTA-AM abolished phase-2 EAD (black filled circle) but not phase-3 EAD (unfilled circles).

**Figure 3.** Phase-3 EAD and V\(_m\) gradient (VG). A, Spatial distribution of phase-3 EADs. V\(_m\) tracings were obtained at sites indicated in the APD map. The blue vertical line denotes the timing for the onset of the largest EAD at the boundary site (site 3). Note a high level of VG at site 3 at this timing. B, Relationship of the amplitude of phase-3 EADs and VG at the EAD onset.

**Figure 4.** Phase-2 EAD-mediated TA. Left panels show snapshots of Ca\(_i\) and V\(_m\) ratio maps at times indicated in the numbers on pseudo-ECG. Right panels show Ca\(_i\) and V\(_m\) tracings at sites indicated on the schematic diagram. Long and short APD regions are shaded and unshaded,
respectively. Red vertical lines denote the QRS onset of the TA. A $V_m$ tracing at the earliest activation site of the TA (site (c)) is shown in blue. Note that propagated phase-2 EADs (black circles) were accompanied by $Ca_t$ re-elevations (red circles), and directly induced the TA at the boundary zone.

Figure 5. Phase-3 EAD-mediated TA. A, Snapshots of $V_m$ ratio maps at times after pacing. B, Phase maps in the same episode. The area where the TA occurred is magnified. C, $V_m$ tracings recorded at sites indicated in the phase map. A blue line shows $V_m$ tracing at the earliest activation site of the TA (site (b)). S = pacing stimulus. D, Superimposed $V_m$ tracings of long APD region (site (a)) and the TA origin (site (b)). VG map just before the TA initiation is also shown. Note a large VG and the presence of phase-3 EADs (unfilled circles) at the site (b).

Figure 6. Interaction between phase-2 and phase-3 EADs. Left panels show snapshots of $Ca_t$ and $V_m$ ratio maps at times indicated in the numbers on pseudo-ECG, phase maps showing the TA initiation (times denote intervals after the onset of escape beat (esc)), and VG map just before the TA initiation. Right panels show $V_m$ and $Ca_t$ tracings recorded at sites indicated in the phase map. Note that phase-2 EAD (filled circle) was only in long APD region (site (a)). At the earliest site of the TA (site (b), blue line), phase-3 EAD (unfilled circle) was present.

Figure 7. Mechanisms of VT. A, Monomorphic VT initiated and maintained by phase-3 EAD-mediated TAs. $V_m$ tracings were recorded at long APD region (site (a)), the origin of the TAs at the boundary zone (site (b)), and short APD region (site (c)). Long APD regions and activation foci are illustrated in schematic diagram as shaded area and circles with arrows, respectively. Note that prominent phase-3 EADs (unfilled circles) were seen only at the boundary zone. Electrotonic transmission of more positive $V_m$ at site (a) to site (b) (antegrade electrotonic interaction, red arrows) may cause the phase-3 EADs, while the resulting TAs may electrotonically depolarize the site (a) (asterisks) by retrograde electrotonic interaction (blue arrows). esc = escape beat. B, Polymorphic VT with varying activation foci. $V_m$ tracings were obtained at long (site (d)) and shorter (site (e)) APD regions. Phase-2 EADs (filled circles) contributed in part to sustenance of long APD region, but was not essential for the VT
maintenance. C, Polymorphic VT with a combined mechanism of focal activation and macro-reentry.

**Figure 8.** Computer Simulations. The mechanisms generating phase-3 EADs in a 1D cable of cardiac myocytes is shown. The 1D cable (200 cells = 1.5 cm) contained two types of cells with different AP types when uncoupled (left traces, black and red, respectively). The cable was paced for one beat from site 1 to induce EADs and TA. A, Short APs (black) and long APs with multiple small phase-2 EADs (red) in a normal \([K^+]_o\) condition. B, APs with short (black) and long (red) AP in a low \([K^+]_o\) condition. Either electrotonic current across steep repolarization gradient or low \([K^+]_o\) caused depolarizing hump consistent with phase-3 EADs (unfilled circles). Note that both effects were necessary for the development of TAs (asterisks) originating from the short APD side of the boundary zone (site 2).
Genesis of Phase-3 Early Afterdepolarizations and Triggered Activity in Acquired Long QT Syndrome

Mitsunori Maruyama, Shien-Fong Lin, Yuanfang Xie, Su-Kiat Chua, Boyoung Joung, Seongwook Han, Tetsuji Shinohara, Mark J. Shen, Zhilin Qu, James N. Weiss and Peng-Sheng Chen

Circ Arrhythm Electrophysiol. published online November 15, 2010;
Circulation: Arrhythmia and Electrophysiology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3149. Online ISSN: 1941-3084

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circep.ahajournals.org/content/early/2010/11/13/CIRCEP.110.959064

Data Supplement (unedited) at:
http://circep.ahajournals.org/content/suppl/2010/11/15/CIRCEP.110.959064.DC1.html

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Arrhythmia and Electrophysiology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Arrhythmia and Electrophysiology is online at:
http://circep.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL for:

Maruyama M, et al.

Genesis of Phase-3 Early Afterdepolarizations and Triggered Activity in Acquired Long QT Syndrome

Supplemental Simulation Methods

The short action potential (AP) model in Fig. 8 panel A is the original rabbit ventricular model.1 The long AP model with EADs was modified from Sato et al2 with the following changes: $G_{ca} = 640 \text{ mmol/(cm C)}$; $G_{to,f} = 0.165 \text{ mS/μF}$; $G_{kr} = 0.05 \text{ mS/μF}$; $G_{ks} = 0.128 \text{ mS/μF}$; $G_{k1} = 0.36 \text{ mS/μF}$; $G_{naca} = 2.94 \text{ μmol/s}$; $P_{ns(ca)} = 3.15 \times 10^{-7} \text{ cm/s}$; $K_{m,ns(ca)} = 0.38 \text{ μmol/L}$; $V_{up} = 0.8 \text{ μmol/ms}$.

The AP in Fig. 8 panel B was modified from the original action potential model1 with the following changes:

Short AP: $[K]_o = 2.7 \text{ mmol/L}$; $G_{to,s} = 0.0012 \text{ mS/μF}$; $G_{to,f} = 0.033 \text{ mS/μF}$; $G_{kr} = 0.0 \text{ mS/μF}$; $G_{ks} = 0.3465 \text{ mS/μF}$; $G_{k1} = 0.06 \text{ mS/μF}$; $G_{nak} = 0.45 \text{ mS/μF}$.

Long AP: $[K]_o = 2.7 \text{ mmol/L}$; $G_{to,s} = 0.02 \text{ mS/μF}$; $G_{to,f} = 0.055 \text{ mS/μF}$; $G_{kr} = 0.0 \text{ mS/μF}$; $G_{ks} = 0.1109 \text{ mS/μF}$; $G_{k1} = 0.12 \text{ mS/μF}$; $G_{nak} = 0.6 \text{ mS/μF}$.

Supplemental References


Supplemental Figures and Figure Legends

Online Supplement Figure I.
Triphenyltetrazolium chloride staining of all sections from a cryoablated heart. The thickness of surviving epicardial layer (stained brick red) was ≈1 mm.
Correlation between APD dispersion and maximal VG during repolarization \((n = 13)\). Individual rabbits are identified by different markers and dashed regression lines. The average within-rabbit correlation coefficient for the relation between APD dispersion and the maximal VG was \(r = 0.91\) (SD 0.09).
Online Supplement Figure III.

Dynamic behavior of the long APD region. A, Cycle length-dependent changes in "island"-like long APD regions. APD maps were obtained at various pacing cycle lengths (PCLs) or during escape rhythm. Although the activation sequences were different during pacing and escape rhythm (not shown), topographical distribution of the long APD islands were similar. B, APD maps (left panels) and $V_m$ tracings at the maximal APD sites (right panels) during constant pacing at different time points. The data were obtained at indicated times after initiation of E-4031 plus low $[K^+]_o/[Mg^{2+}]_o$. As time goes on, the APDs tend to be longer with a minor (heart #1) or greater (heart #2) change in the spatial pattern of the APD.
Online Supplement Figure IV.
Cycle-length dependence of the amplitude and coupling interval of phase-3 EADs (n = 6). The amplitude of phase-3 EAD is the $V_m$ difference between the diastolic $V_m$ and the first deviation from the contour during phase-3 repolarization. The coupling interval of phase-3 EAD is the time from phase-0 upstroke to the time at which the first deviation occurs. The amplitudes of phase-3 EAD were significantly different among different pacing cycle lengths (PCLs). ($P < 0.001$ by the overall ANOVA repeated measure test)
Online Supplement Figure V.

Simultaneous recordings of optical $V_m$ (blue lines) and TMPs (black lines). Optical $V_m$ tracing was obtained in the immediate vicinity of the TMP recording site. A, Action potential recordings during a single TA beat at long and short APD regions. Note almost identical time-course between TMP and optical $V_m$ despite slower upstrokes in optical $V_m$. B, TMP and optical $V_m$ recordings in the long APD region near the boundary zone during VT. Note phase-2 (filled circles) and phase-3 (unfilled circles) EADs in both recordings.
Online Supplement Figure VI.

Phase-2 EADs (filled circles) and phase-3 EADs (unfilled circles) in the absence (A) and presence (B) of TAs. $V_m$ optical signals were recorded at sites indicated in the numbers and alphabets on the APD map and isochronal maps for the TAs, respectively. The blank area on the isochronal map denotes the long APD region. Spatial and temporal distributions of phase-2 and phase-3 EAD are distinct, which made it straightforward to differentiate each type of EAD. We determined which phase of EAD induced a TA by the type of EAD observed at the earliest activation site of the TA beat. Examples of TA mediated by phase-2 EAD (filled asterisk) and phase-3 EAD (unfilled asterisk) are shown. Note that the repolarization of the triggering beat (stimulated (S) or escape (esc) beat) is interrupted at various $V_m$ levels by the propagated TA activation; therefore, responsible EADs can only be defined by detailed mapping, not by local take-off potentials of the TA.
Online Supplement Figure VII.

Torsades de pointes (TdP). A, VT with TdP-like ECG morphology. B shows another episode of TdP in which we have optical maps available. There was phase-2 EAD, which mediated phase-3 EAD through electrotonic interaction. Ca$_i$ (red line) and V$_m$ (black lines) signals were recorded at sites indicated in the V$_m$ ratio map. The TdP started 368 ms after the onset of an escape beat (esc). The long APD region was noted in the base of the RV and mid part of the LV 4 ms before the initiation of TdP. Focal Ca$_i$ re-elevation (red circle, red arrowhead in Ca$_i$ ratio map) induced phase-2 EAD (black circle) at site (a), which in turn enhanced VG across the site (a) and (b). Phase-3 EAD (unfilled circle) developed at site (b) and induced a TA leading to a spiral reentry. White circles in the phase maps during the TdP denote phase singularity (PS) (i.e. spiral core) and the schematic diagram illustrates trajectory of the PS, which shows drifting the spiral core.
Legends for the Video Files

**Online Movie I.** $V_m$ ratio map during monomorphic VT originating from a single focus at the boundary zone. The movie covers 2.5 second of the data. The movie starts with an escape beat. Following beats represent VT.

**Online Movie II.** $V_m$ ratio map during polymorphic VT with multiple foci in the boundary zone. The movie covers 2.8 seconds of the data. The first beat is an escape beat. Note that the size and shape of the long APD region and the VT focus altered from beat to beat, resulting in the polymorphic appearance of the QRS complex.

**Online Movie III.** $V_m$ ratio map during polymorphic VT with reentry and focal activations. The movie covers 2 second of the data. Following an escape beat, multiple focal activations induced macro-reentry. The reentrant wavefront propagated around the long APD region in a counterclockwise direction. Focal activation was responsible for the final VT beat.