Title
The Dynamical Emergence of Calcium-Mediated Arrhythmias from the Subcellular Calcium Release Unit Network

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Publication Date
2016

Peer reviewed|Thesis/dissertation
The Dynamical Emergence of Calcium-Mediated Arrhythmias
from the Subcellular Calcium Release Unit Network

A dissertation submitted in the partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Molecular, Cellular, and Integrative Physiology

by

Christopher Young Ko

2016
ABSTRACT OF THE DISSERTATION

The Dynamical Emergence of Calcium-Mediated Arrhythmias from the Subcellular Calcium Release Unit Network

by

Christopher Young Ko

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

University of California, Los Angeles, 2016

Professor James N. Weiss, Chair

Abnormal calcium (Ca) cycling conditions in cardiac myocytes have been identified as a major contributor to lethal cardiac arrhythmias that lead to sudden cardiac death. Under these conditions, Ca waves can emerge spontaneously from within the subcellular Ca release unit (CRU) network in Ca overloaded myocytes and facilitate the formation of delayed afterdepolarization (DAD) and early afterdepolarization (EAD) arrhythmia triggers at the cellular scale. Whether DADs and EADs can overcome the electronic sink effect of resting neighboring myocytes to propagate and disrupt the normal conduction of action potentials in cardiac tissue highly depends upon the timing and spatial organization of myocytes exhibiting spontaneous Ca waves. The exact mechanisms that govern these events are still under much debate and not fully understood. The goal of our studies in this body of work is two-fold. The first is to understand how a broad spectrum of spontaneous Ca release events, including Ca waves, are able to emerge collectively from within the CRU network. The second is to understand how the effects of subcellular Ca release events traverse scales to influence the morphology of DADs and EADs at the cellular scale and promote arrhythmias at the tissue scale. In order to achieve
these ends, we have utilized a multidisciplinary approach that draws upon and integrates ideas, concepts, and techniques largely from experimental electrophysiology, nonlinear dynamics, and mathematical modeling. This approach has allowed tremendous versatility in the way arrhythmias can be conceptualized, quantitatively assessed, and logically predicted. As such, we have discovered a universal mechanism of criticality that governs the transition from stochastic Ca sparks to deterministic Ca waves, a general theory for Ca wave entrainment that can explain subcellular pacemaker site emergence, novel complex EAD morphologies that emerge due to the interaction between the stochastic effects of subcellular Ca release and the deterministic relationships among voltage-gated ion channels, that the distribution of latencies to the onset of Ca waves among myocytes is the most important factor promoting DADs in cardiac tissue, and that DADs and EADs can promote arrhythmias not only by generating triggers but by enhancing tissue substrate vulnerability.
The dissertation of Christopher Young Ko is approved.

Zhilin Qu

Kenneth D. Philipson

Jau-Nian Chen

James N. Weiss, Committee Chair

University of California, Los Angeles

2016
I dedicate this thesis to my family
for their endless love, dedication, and support
and
to my friends, colleagues, and teachers
who have taught me something new every day.
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<tbody>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>AV node</td>
<td>Atrioventricular node</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium-induced calcium release</td>
</tr>
<tr>
<td>Cl</td>
<td>Chlorine</td>
</tr>
<tr>
<td>CRU</td>
<td>Calcium release unit</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAD</td>
<td>Delayed afterdepolarization</td>
</tr>
<tr>
<td>EAD</td>
<td>Early afterdepolarization</td>
</tr>
<tr>
<td>ECC</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>ICa,L</td>
<td>L-type calcium current</td>
</tr>
<tr>
<td>IK1</td>
<td>Inward rectifier potassium current</td>
</tr>
<tr>
<td>ICaX</td>
<td>Sodium-calcium exchange current</td>
</tr>
<tr>
<td>IKC</td>
<td>Calcium-activated potassium current</td>
</tr>
<tr>
<td>PKA</td>
<td>Phosphokinase A</td>
</tr>
<tr>
<td>PLB</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>PVC</td>
<td>Premature ventricular contraction</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SA node</td>
<td>Sinoatrial node</td>
</tr>
<tr>
<td>SCD</td>
<td>Sudden cardiac death</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum</td>
</tr>
<tr>
<td>LTCC</td>
<td>L-type calcium channel</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium-calcium exchanger</td>
</tr>
<tr>
<td>PAC</td>
<td>Premature atrial contraction</td>
</tr>
<tr>
<td>PKA</td>
<td>Phosphokinase A</td>
</tr>
<tr>
<td>PLB</td>
<td>Phospholamban</td>
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<td>SERCA</td>
<td>Sarco/endoplasmic reticulum</td>
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<td>ATP-ase</td>
<td>ATP-ase</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TA</td>
<td>Triggered activity</td>
</tr>
<tr>
<td>VF</td>
<td>Ventricular fibrillation</td>
</tr>
<tr>
<td>VT</td>
<td>Ventricular tachycardia</td>
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ACKNOWLEDGEMENTS

My years of graduate study have been some of the most fruitful in my intellectual development and growth as a scientist. I was fortunate to have been surrounded by brilliant minds and tremendous talent in a setting at UCLA ideal for training in the biomedical sciences. Accordingly, the body of work described in this dissertation was a collaborative effort involving support and assistance from many generous people.

First and foremost, I would like to thank my mentor Dr. James N. Weiss. He has been my scientific role model since the first time I met him, and I have been inspired in many ways by his genuine passion for science and his insatiable hunger for understanding not only the heart but also the natural world at large. It is to him that I owe a great portion of the credit for my scientific growth. From the way he thinks and integrates knowledge from a diverse spectrum of fields to the way he coherently conveys that knowledge to others, I have tried to emulate each of the traits which I believe has not only brought him success as a physician scientist but makes him one of the most brilliant thinkers I have ever met. I also would like to thank Dr. Weiss for creating a research and learning environment that allowed freedom of scientific inquiry and for his patience and guidance as I honed the skills necessary for achieving a successful future career in science. If the environment were not as such, I would not have been able to learn or grow nearly as much as I have in the last several years. I consider it a privilege to have felt happy to wake up every morning knowing I would be doing what I love to do.

I also owe great gratitude to Dr. Weiss and his leadership for cultivating a community of motivated scientists, physicians, professors, trainees, and staff within the Cardiovascular Research Laboratories (CVRL) and the Division of Cardiology. It did not take me long to realize that each person Dr. Weiss had recruited within his arsenal of talent was someone who was extraordinary in his or her own right. Thus, I would like to give special recognition to Dr. Thao P. Nguyen who not only taught me the technical skills for patch clamp electrophysiology but also
valuable advice stemming from her own personal experiences that has helped me to gain confidence and navigate my way as a trainee in our research group. In my later years, I have had the privilege of passing on what I have learned from her to the new and young trainees in her own lab. Another member in the Weiss lab to whom I owe much thanks is Dr. Guillaume Calmettes. In addition to French, he has introduced me to the coding language of Python, which has revolutionized the way I analyze, present, and share data. I thank him for his subtle encouragements and for being a partner on the quest for improved implementation of statistics in biology. Other members in the Weiss lab I would like to thank include Pauline Morand, Shuzhen Zhang, Rahil Patel, and Nicole Petrochuk for their dedication and expertise in cardiac myocyte isolations. I would also like to thank Alfonso Lopez and Raul Moreno, David Ayvazian and Tan Duong, and John Parker for their willingness to lend a hand and keep an eye out for my needs pertaining to administrative, computer, and engineering and equipment issues, respectively. When the going got tough, I also had the privilege of turning to senior members in the lab. I would like to express my thanks to Dr. Scott John (not John Scott) for hearing out my inquiries and for introducing me to the world of whisky (without the e), to Dr. Tom Yang for brainstorming solutions to technical roadblocks in patch clamping, and to Dr. Paavo Korge for his kind greetings and library of metabolic reagents. Among the fellow trainees in the lab who have come or gone during my time, I would like to thank Dr. Aneesh Bapat, Dr. Marvin Chang, and Dr. Jonathan Hoffman and Vanitra Richardson for being a part of my journey.

The credit for the work I accomplished extends far beyond the borders of our laboratory. The strength of our arrhythmia biology studies, in fact, lies in the extraordinary collaboration among the research groups in the arrhythmia biology Program Project Group. All of the research described here has been performed under the direction of the group’s senior members Dr. James N. Weiss, Dr. Zhilin Qu, Dr. Alan Garfinkel, Dr. Riccardo Olcese, Dr. Hrayr Karagueuzian, Dr. Peng-Shen Chen, and Dr. Thao P. Nguyen. Each lead investigator and his or
her respective team brought unique perspectives and approaches to understanding and solving arrhythmia problems.

Dr. Zhilin Qu was the mastermind behind many of our theories in nonlinear dynamics and theoretical physics as they applied to this body of work. He brings some of the most novel ideas to the table in our discussions and is highly respected by colleagues and trainees alike. For all of my projects, I have had the privilege of working closely with trainees from Dr. Qu’s research group. Special recognition and thanks go to Dr. Michael Nivala, Dr. Zhen Song, and MSTP student Michael Liu who have been the mathematical modeling counterparts to my experimental roles in each of our co-authored works. Without their collaboration, none of the work presented here would have come to fruition. Furthermore, Dr. Enno de Lange is a former trainee from the Qu group who I would like to thank for teaching me the fundamentals of nonlinear dynamics in a way that was readily understandable and applicable. Thanks also go to those who have contributed to my learning in our meetings including Julian Landaw.

When it comes to the principles of applying mathematics to biology, I must undoubtedly thank Dr. Alan Garfinkel who has an uncanny gift of clearly conveying very difficult concepts in just about the most understandable and simplest ways possible. I have thoroughly enjoyed each one of his lectures and seminars which were always vivid, memorable, and informative. His presentations never failed to leave me inspired and genuinely excited to conduct scientific research. Thanks also go to his trainee, Imesh Samarakoon, who has aided my learning through his contributions in our meetings.

Dr. Riccardo Olcese is an expert in experimental electrophysiology who always expressed a contagious enthusiasm for solving problems in biophysics. I felt inspired by the way he approached problems and amazed by the solutions he found in solving very difficult problems. In addition to the dedication to his work, his genuine interest in mentoring and fostering the growth of his trainees was admirable and evident to all. I thank him for his cheerful demeanor and always making me feel included as a trainee in our collaboration. I have also had
the privilege of being student colleagues with his former trainee, Dr. Roshni Madhvani, who I have admired for her ability to articulate scientific findings clearly to all audiences. I would like to thank her for sharing along my scientific training journey. I would also like to acknowledge Dr. Marina Angelini, another trainee in the Olcese group, with whom I have also shared in the challenge of patch-clamping isolated cardiac myocytes.

Dr. Hrayr Karagueuzian and his team of former and current trainees, including Dr. Jay Choi and Dr. Arash Pezhouman, have set a standard of excellence in experimental whole-heart electrophysiology and imaging recordings. Over the years, I am appreciative of having learned from Dr. Karagueuzian the value of considering history and garnering appropriate respect for the work of those who have set the groundwork for science today.

Dr. Peng-Shen Chen from the University of Indiana has provided valuable perspectives not only pertaining to the experimental but to the clinical as well. I have enjoyed interacting with members from his research group on their visits and look forward to their updates during our teleconference meetings.

Finally, there are several trainees in Dr. Thao Nguyen’s research group whom I have encountered that I would like to thank for their role in helping me exercise and impart some of the practical knowledge and skills I have garnered during my training period. They include Dr. Neha Singh, Shankar Iyer, Chinwe Mbisike, Kalissa Genevieve Van Cleave, Himani Madnawat, Minu Madhvani, Kayla Mayaki, Mojdeh Dooraghi, Dr. Li Jie, Dr. Binh Nguyen, Nicole Nguyen, Brian Nguyen, Beshoy Sami, and Maryam Tavanaei.

In the winter of 2012, I have had the opportunity to visit Charleston, South Carolina to learn how to implement a rapid solution delivery technology for patch clamp electrophysiology. I would like to thank Dr. Martin Morad and Dr. Angelo Oscar da Rosa for teaching me how to make and use the “puffer.”

My graduate student experience has truly been one of the most enjoyable seasons along my academic journey. Therefore, I would like to thank Dr. James Tidball for his leadership.
as the Chair of the Molecular, Cellular, and Integrative Physiology Interdepartmental Program (MCIP IDP) during my time as a doctoral student. His support and genuine care for the welfare of the students has been unparalleled, and it truly is not an exaggeration when his dedication to the program has been described as a labor of love. Today, the program is a collection of talented faculty and students, and MCIP now stands as one of the most prestigious graduate training programs in physiology in the country. Among my student colleagues, I would like to give special recognition to Rory Spence, to Diana Tran, who had come before me also as a trainee in the arrhythmia biology group, and to Dr. Charles Hummel, Dr. Jessica Gluck, Dr. Roshni Madhvani, and Dr. Quan Li with whom I began my journey in the program. Furthermore, my success in the MCIP doctoral program would not have been possible without the basic science skills and knowledge I gained in the UCLA Physiological Master's program. Therefore, I would like to thank my former advisor Dr. Rachelle Crosbie-Watson for her mentorship during my Master's studies.

As my journey in MCIP nears its end, I would like to extend my thanks to Dr. Zhilin Qu, Dr. Kenneth D. Philipson, Dr. Jau-Nian Chen, and Dr. James N. Weiss for their time, patience, and support while serving on my thesis committee.

Lastly, I would like to thank my parents and my brother for being my examples for what it means to work diligently, faithfully, and with hope even in the face of uncertainty.

Several of the chapters in this body of work are reprints of work published with colleagues in our research group. Chapter 2 contains a reprint (under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License) of “Criticality in Intracellular Calcium Signaling in Cardiac Myocytes” co-authored by Michael Nivala, Christopher Y. Ko, Melissa Nivala, James N. Weiss, and Zhilin Qu and published in the Biophysical Journal in June, 2012 (102(11):2433-42; doi: 10.1016/j.bpj.2012.05.001). Chapter 3 contains a reprint (with permission from John Wiley and Sons) of “The emergence of subcellular pacemaker sites for calcium waves and oscillations” co-authored by Michael Nivala, Christopher Y. Ko, Melissa
Nivala, James N. Weiss, and Zhilin Qu and published in the Journal of Physiology in November, 2013 (591:5305-5320; doi: 10.1113/jphysiol.2013.529960). Chapter 4 contains a reprint (with permission from Elsevier) of “Calcium-voltage coupling in the genesis of early and delayed afterdepolarizations in cardiac myocytes” co-authored by Zhen Song, Christopher Y. Ko, Michael Nivala, James N. Weiss, and Zhilin Qu in the Biophysical Journal in April, 2015 (108(8):1908-21; doi: 10.1016/j.bpj.2015.03.011). Chapter 5 contains a modified manuscript in preparation for publication entitled, "From Ca Waves to Triggered Activity in Heart: Scaling from the Subcellular to the Tissue Level" and co-authored by Christopher Y. Ko, Michael B. Liu, Zhen Song, Zhilin Qu, and James N. Weiss. In each of the respective works, my main contributions pertain to the experimental aspects, the main contributions of Michael Nivala, Melissa Nivala, Zhen Song, and Michael B. Liu pertain to the computational modeling aspects, and Zhilin Qu and James N. Weiss have been the principle investigators.

Some images used in the figures were found online. In Figure A.1, the sand ridge, river tributary, hurricane, and ridge patterns on fingertips images are in the public domain; the fish skin pattern and the circulatory vasculature images (under the Creative Commons Attribution 3.0 Unported license) are attributed to CSIRO and Thomasjst, respectively; the tree branches image and the nautilus shell image (under the Creative Commons Attribution-Share Alike 3.0 Unported license) are attributed to Hermux and Chris 73, respectively; and the cochlea image (under the Creative Commons Attribution-NonCommercial-No Derivatives 4.0 International license) is attributed to Dr. David Furness. All other images used in the figures were acquired, generated, or created personally or by colleagues and used with permission.

This dissertation was financially supported by National Institutes of Health grants P01 HL078931, R01 HL103622, R01 HL110791, R56 HL118041, a research grant from Gilead Sciences, a postdoctoral fellowship award from the American Heart Association Western States Affiliate 10POST3210024, a predoctoral fellowship award from the American Heart Association Western States Affiliate 10PRE3030052, Molecular, Cellular, and Integrative Physiology
Interdepartmental Program NIH Training Fellowship T32 GM065823, UCLA MSTP Grant T32 GM008042, the UCLA Cardiovascular Development Fund, and the Laubisch and Kawata endowments.
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Introduction and general background
1.1 A brief historical overview

The heart is one of the most intriguing and complex organs in the human body. For millennia, it has fascinated and sometimes even mystified those who have had vested interest in understanding its function both in health and in disease. Some of the earliest records of the heart and its diseases can be found in the Ebers papyrus, written by ancient Egyptians during the pharaonic period.

As to ‘the heart weakens,’ it means the heart does not speak, or it means the [peripheral vessels] are dumb. There is no remedy for them or information under your two hands, which normally appears because of the air with which they are filled.

In ancient Egypt, much metaphysical significance was attributed to the heart, which was often personified, as it was considered to be the seat of emotions. Moreover, malign supernatural influences were believed to cause many diseases, and the physician’s role was to invoke benign deities to counteract the malign influences. It is important to note that the understanding of the heart held by the ancient Egyptians, as well as the view of the influence of magic and the supernatural on medicine and disease, were consistent with the prevailing sets of beliefs of the day. As fragmentary records and literary epics such as those written by Homer indicate, other civilizations, such as Mesopotamia and ancient Greece of the Heroic Age, also shared these views.

Across civilizations and throughout history, the views and understanding of the heart, and medicine in general, evolved much in accord with the prevailing traditions of a given era. However, there would oftentimes come periods in history in which new perspectives and frames of thought capable of reconciling contradictions and overcoming limitations in the prevailing tradition would dramatically redirect the course of the pursuit of understanding and knowledge. Entering the Hellenistic Age in ancient Greece, traditions in philosophy, established in part by the likes of Alcmaeon, Hippocrates, Herophilus, and Galen, led to the view that humans as well
as disease are part of the natural world that is subject to laws of cause and effect that operate within and at large. This fundamental shift in perspective, free from the influence of magical and religious elements, allowed for rational modes of explanation, formal deductive reasoning, and logical argument to become an integral part of medicine. Following the Middle Ages, rediscovery of classical Greek philosophy and the rise of humanism during the Renaissance set the groundwork for modern medicine to take root. Sir William Osler, considered by many to be the father of modern medicine through its development during the 19th and 20th centuries, largely attributed progress during the Renaissance to three individuals, as follows:

The sixteenth and seventeenth centuries did three things in medicine—shattered authority, laid the foundation of an accurate knowledge of the structure of the human body and demonstrated how its functions should be studied intelligently—with which advances, as illustrating this period, may be associated the names of Paracelsus, Vesalius and Harvey.

The discovery of the circulation of blood by William Harvey in 1616 is considered to mark the beginning of cardiology. Over the next several hundred years, cardiology developed much in step with the establishment of biology, chemistry, and physics as branches of classic modern day science as we know them today. The path cardiology roughly followed encompassed pathology and descriptive anatomy in the 17th and 18th centuries, auscultation and its correlations in the 19th and 20th centuries, and from the turn of the 21st century, major advances in diagnosis and treatment of heart disease driven by technology, which includes precision instruments to measure blood pressure, the electrocardiogram, clinical catheterization, and pharmacology. Today, cardiology is organized into multiple, highly specialized disciplines in the realms of clinical practice as well as of research in the clinical and basic science settings. In particular, genomics and molecular biology, and their diagnostic and therapeutic applications, including cell therapy approaches in cardiac stem cell biology, have come to the forefront.
Despite the long and illustrious history of the study of the heart, the mission to understand the heart and to prevent and treat disease has been formidable. Cardiovascular disease (CVD) is the leading cause of death and disability today in not only the developed world but also in a majority of the developing world as well. The global burden is truly at epidemic proportions, with the World Health Organization attributing a third of all deaths worldwide, or an estimated 17 million lives, to CVD each year. CVD takes on many forms, including coronary heart disease, myocardial infarction, hypertension, and congestive heart failure. Among them all, sudden cardiac death (SCD) is the most prevalent and is responsible for half of all CVD deaths, including the lives of more than 300,000 in the United States alone each year. Of all SCD events, ventricular fibrillation (VF), a particularly lethal form of cardiac arrhythmia, is responsible for the majority.

Cardiac arrhythmias have been observed as abnormalities in heart rhythm or rate even since ancient times. What is perhaps the earliest description of cardiac arrhythmias can be found in the Ebers papyrus as follows:

“When the heart is diseased, its work is imperfectly performed: the vessels proceeding from the heart become inactive, so that you cannot feel them… if the heart trembles, has little power and sinks, the disease is advanced and death is near.”

Today, the problem of cardiac arrhythmias, including some of the most common forms such as atrial fibrillation and ventricular tachycardia (VT) and fibrillation, remains largely unresolved. Despite dramatic improvements that have been made in the diagnosis and treatment of arrhythmias in the last century, clinical interventions so far have demonstrated only moderate success overall. The most successful interventions have primarily involved the application of engineering strategies, including the use of pacemakers, implantable cardioverter-defibrillators (ICDs), and catheter ablation techniques. Among these, ICDs have likely been the most effective. However, ICD implementation has been suboptimal largely due to the difficulty of predicting which patients are at greatest risk. In fact, 80% of the 300,000 patients who die
suddenly each year do not have clinical indications for ICD placement\(^9\). Moreover, only 25% of the patients who do receive ICD implantation incur life-saving shocks, which prolong lives only modestly at 4.4 months\(^10\). Among the engineering approaches, antiarrhythmic ablation techniques have also proved to be of moderate success. First introduced by surgeons in the 1970s, ablation techniques were designed to physically interrupt arrhythmogenic conduction circuits by directly freezing or cutting away abnormal tissue. Today, catheter ablation techniques are curative for many supraventricular arrhythmias. However, they are yet limited in the many arrhythmia scenarios that do not involve structural tissue abnormalities. Among pharmacologic and biologic approaches to treating arrhythmias, no method has yet emerged with comparable efficacy to the ICD. Yet, the pharmacological approaches which have proven to be more successful and reduce the incidence of SCD in large cohorts of patients with heart failure and ischemic heart disease include the use of β-adrenergic receptor blockers, angiotensin-converting enzyme (ACE) inhibitors, and aldosterone antagonizers\(^11\). Therapeutic strategies that have sought to directly target ion channels and inhibit their activity have yielded complicated outcomes largely due to the fact that the channel functions being inhibited are often critical components for normal cardiac function.

1.2 Cardiac arrhythmias

The main area of investigation in this body of work is on cardiac arrhythmias. In order to convey the nature of the problem of cardiac arrhythmias, it is apt to present an overview of the basic physiology of the heart and the current understanding of arrhythmogenesis.

1.2.1 Basic physiology of the heart

In each of the approximately 3 billion times the human heart beats within the human lifespan, the four chambers of the heart – the right atrium, right ventricle, left atrium, and left
Figure 1.1. Whole-heart physiology

A. The cardiac cycle. During systole, atrial contraction is followed by ventricular contraction. The right atrium pumps deoxygenated blood (blue arrows) coming from the systemic circulatory system into right ventricle, which pumps blood to the lungs. The left atrium pumps oxygenated blood (red arrows) coming from the lungs to the left ventricle, which pumps blood to the systemic circulatory system. During diastole, all chambers of the heart relax and refill with blood.

B. Cardiac electrical conduction system. Electrical impulses called action potentials conduct across the electrically excitable syncytium of myocardial tissue and coordinate the contraction of both the atria and ventricles. Action potentials (black arrows) originate at the sinoatrial node and propagate as waves across the atria until they reach the atrioventricular node. From there, they conduct rapidly through the His-purkinje system and rapidly excite the ventricular myocardium. Extra-systolic electrical excitations (red star) that originate abnormally from regions other than the sinoatrial node can act as arrhythmia triggers that disrupt normal cardiac conduction and contraction and lead to arrhythmias.

The heart is a particularly unique organ in that the rod-shaped cells in the heart, called myocytes, are electrically excitable and physically coupled to form a syncytium of excitable tissue. This arrangement allows for electrical impulses called action potentials (APs) (Figs. 1.1B, 1.2A) to propagate as waves across cardiac tissue and, in doing so, coordinate the sequence of
contraction of the chambers. During normal conduction, a wave of APs originating at the sinoatrial node (SA node), the heart’s pacemaker located in the right atrium, propagates across the right and left atria to the atrioventricular node (AV node). From the AV node, the APs rapidly conduct through the quasi 1-dimensional network of the His-Purkinje system and then to both chambers of the ventricles. Since the timing and sequence of AP propagation across the myocardium is critical for coordinating contractions, disruptions in the normal initiation, timing, or conduction of APs can cause arrhythmias. Therefore, cardiac arrhythmias can be defined generally as abnormal changes in heart rate or rhythm due to disruptions in the coordinated conduction of electrical impulses in the heart. Severe loss of coordinated contraction, as occurs during extreme cases such as VF, can result in SCD.

1.2.2 Arrhythmia triggers and vulnerable tissue substrate

Arrhythmogenesis involves arrhythmia triggers. Common arrhythmia triggers include premature atrial contractions (PACs) and premature ventricular contractions (PVCs), which can emerge due to ectopic foci, or regions of cardiac tissue other than the SA node from which APs may initiate between normal sinus beats. PACs and PVCs are not uncommon in the normal heart and are generally benign. Even in many diseased heart contexts that have higher frequencies of PAC and PVC, which occur on a time scale of minutes and hours, SCD episodes are infrequent and occur on a time scale of months or years. In order for arrhythmias to emerge, the arrhythmia trigger needs to encounter a tissue substrate vulnerable to the initiation and maintenance of sustained arrhythmias. At the tissue or organ scale, arrhythmia triggers as well as the vulnerability of the tissue substrate classically have been attributed to pre-existing tissue heterogeneities, which are the intrinsic regional electrophysiological and structural differences often exacerbated in the diseased heart by electrical, structural, vascular, and neural remodeling processes. Hence, arrhythmias are commonly observed to initiate in such areas as
border zones around the pulmonary veins in atrial tissue or around areas of ischemic injury or fibrosis in ventricular tissue.

1.2.3 Cellular electrophysiology

As with skeletal myocytes and neurons, APs are produced in cardiac myocytes at the cellular level due to ion channels and pumps that function in context of an electrochemical gradient across the cell membrane. Accordingly, the electrophysiology of the myocyte significantly influences the manner in which the heart functions. At rest under normal conditions, the electrical potential maintained in the myocyte across the cell membrane is approximately -80 mV relative to the extracellular environment. During the excitatory phase of the AP, sodium (Na) and calcium (Ca) are two major ions that move into the myocyte to produce inward currents that depolarize, or make more positive, the membrane potential. During the recovery phase of the AP, potassium (K) is the major ion that moves out of the cell to produce outward currents that repolarize the membrane potential back to resting levels. Chloride (Cl) currents, to a lesser extent, are also involved in repolarization. The specific amounts and timing of these ions crossing the cell membrane are critical in shaping the normal AP. Therefore, altered ion channel function, such as those associated with channelopathies, or perturbations in the balance or timing of currents across the membrane can increase the risk of arrhythmias.

1.2.4 Early afterdepolarizations and delayed afterdepolarizations

Early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs) are two abnormal patterns of excitation at the cellular level classically associated with arrhythmia triggers. Of these two, EADs typically appear as oscillations in the depolarization phase of the AP (Fig. 1.2B) and are caused by a combination of negative feedback and time delay interactions between the inward L-type Ca current ($I_{Ca,L}$) and outward K currents. Furthermore,
**Figure 1.2. Myocyte Electrophysiology**

**A. The cardiac action potential.** The cardiac action potential (black trace) represents whole-cell changes in myocyte voltage due to the flux of mainly Na, Ca, K, and Cl ions across the sarcolemma membrane. The Ca transient (green trace) represents whole-cell changes in intracellular Ca during the action potential.

**B. Early afterdepolarizations.** Changes in the magnitude or timing of myocyte currents can produce oscillations called EADs in the plateau phase of the action potential. Whole-cell intracellular Ca levels can vary in step with voltage changes in the myocyte.

**C. Delayed afterdepolarizations.** Spontaneous SR Ca release that activates $I_{NCX}$ and other non-selective ion currents can produce transient depolarizations in the resting phase of the action potential called DADs. DADs that are sufficient in amplitude to activate $I_{Na}$ can trigger extra-systolic action potentials called triggered activity.

EADs typically occur in the setting of reduced repolarization reserve, which is caused by reductions in outward currents (e.g. $I_{K1}$), increases in inward currents (e.g. $I_{Ca,L}$, $I_{NCX}$, $I_{Na}$), or both. DADs, on the other hand, typically appear as transient depolarizations of the resting membrane potential between APs (Fig. 1.2C) and are caused by spontaneous intracellular Ca release events that activate the Na-Ca exchange current ($I_{NCX}$) and other non-selective ionic currents. DADs are typically potentiated by conditions of Ca overload and, if sufficient to reach the Na current ($I_{Na}$) excitation threshold, can generate ectopic APs called triggered activity (TA).
1.3 Calcium

1.3.1 Calcium cycling and excitation-contraction coupling

Of the major ions involved in the electrophysiology of the myocyte, Ca plays critical roles in many molecular processes at the subcellular level. As such, a Ca cycling process regulates the amount of Ca present within the myocyte from beat to beat (Fig. 1.3A). This process begins with myocyte depolarization and the subsequent activation of voltage-gated L-type Ca channels (LTCCs) located in invaginations in the cell membrane called t-tubules. Extracellular Ca then enters the myocyte and, in a process called Ca-induced Ca release (CICR), binds and activates ryanodine receptors (RyRs) to trigger the release of a larger store of Ca from the lumen of the sarcoplasmic reticulum (SR). At this point, intracellular Ca is available to enable several molecular processes. In excitation-contraction coupling (ECC), Ca acts to release

Figure 1.3. Calcium signaling in cardiac myocytes
A. Calcium cycling. Ca is cycled on every beat in cardiac myocytes. Ca cycling begins as extracellular Ca enters the myocyte through L-type Ca channels (LTCCs). Via the mechanism of Ca-induced Ca release (CICR), Ca entering the myocyte via LTCCs activates ryanodine receptors (RyRs), which release a large store of Ca from the sarcoplasmic reticulum (SR). Ca from LTCCs and RyRs diffuse rapidly into the cytoplasmic space and is available to initiate contraction (i.e. excitation-contraction coupling) and other Ca-mediated processes. Ca extrusion from the myocyte via the Na-Ca exchanger (NCX) primarily and Ca resequestration back into the SR via the serca/endoplasmic reticulum ATP-ase (SERCA) pumps bring intracellular Ca back to resting levels and completes the Ca cycling process.

B. The calcium release unit network. The calcium release unit (CRU) or couplon is composed of LTCCs at the sarcolemma membrane adjoined to RyRs on the SR membrane. CRUs distributed across the network of t-tubules (blue lines) within the myocyte form the subcellular CRU network.
tropomysin from actin thin filaments, which allows for actin-myosin interactions that enable shortening of sarcomeres, the molecular units of contraction. Hence, Ca is the key element that links the heart’s electrical excitations to its mechanical contractions. During the AP, Ca also can act to modulate ion channel function through ligand-mediated interactions such as those that occur in LTCC inhibition, in enhancing RyR activation, and in Ca-activated K channel current ($I_{SK}$) activation. During repolarization, the Ca cycling process comes to completion and intracellular Ca returns to baseline levels as Ca is extruded from the myocyte through NCX and also sequestered back into the SR via sarco/endoplasmic reticulum Ca-ATPase (SERCA) pumps on the SR membrane.

1.3.2 The role of calcium in molecular pathways

When Ca levels remain persistently elevated under conditions such as rapid heart rates, autonomic nervous system regulation, or increased RyR leakiness, several longer-term changes can occur due to the activation of a myriad of signaling pathways. Several of these can be described beginning with the Ca-mediated activation of calmodulin. When Ca binds calmodulin, CaMKII is able to phosphorylate and enhance the activity of LTCCs, RyRs, and SERCA. CaMKII can also phosphorylate phospholamban (PLB), which in turn loses its inhibition on SERCA and enables enhanced SERCA activity. Ca-bound calmodulin can also facilitate positive feedback effects that ultimately work to increase intracellular Ca by activating adenylyl cyclase (AC). AC activation leads to increased production of cAMP, which in turn activates protein kinase A (PKA), the kinase responsible for phosphorylating LTCCs, RyRs, and PLB. In general, molecular changes that enhance the rates of depolarization and repolarization or the rates of intracellular Ca cycling can shorten the periodicity of Ca cycling and increase the heart's pacing rate. At the same time, high basal levels of phosphodiesterases and
phosphoprotein phosphatases, which regulate PKA and CaMKII activity\textsuperscript{19}, can mitigate these effects.

1.3.3 The subcellular calcium release unit network and the calcium signaling hierarchy

Just as the myocytes in the heart are physically coupled to form a syncytium of excitable tissue capable of generating propagating waves of APs, the subcellular Ca cycling molecules are coupled by diffusive Ca to form an excitable intracellular network capable of generating propagating waves of Ca. In an average myocyte, this network is composed of approximately 20,000 individual Ca release units (CRUs) (Fig. 1.3B), or couplons, each composed of about 10 LTCCs in the t-tubules of the myocyte membrane apposed to about 100 RyRs in the SR membrane\textsuperscript{21}. Within the CRU network, each CRU is capable of producing the Ca spark, which is

Figure 1.4. Calcium signaling hierarchy. The CRU network gives rise to a wide spectrum of Ca release behaviors predicted by the “3R theory,” which accounts for the randomness of CRU firing, the refractoriness of CRUs, and diffusive Ca-mediated recruitment of neighboring CRUs. Common Ca release behaviors include the Ca spark, classically considered to be the unitary form of Ca release, macrosparks (spark clusters), semi-propagating abortive waves, fully propagating waves, and waves of more complex morphology such as spiral waves.
classically considered to be the elementary unit of Ca release\textsuperscript{22}. Ca sparks can be produced in several ways. Those that occur due to activation of LTCCs or by spontaneous activation of RyRs are called “primary sparks”\textsuperscript{23}. Those that occur due to opening of RyRs in a CRU in response to Ca released from neighboring CRUs are called “secondary sparks” or “spark-induced sparks”\textsuperscript{23}. Depending on the relative probabilities of occurrence of primary and secondary sparks and the spatiotemporal summation of multiple Ca sparks, the CRU network can give rise to the classical Ca signaling hierarchy\textsuperscript{24} (Fig. 1.4), which ranges from Ca quarks (single RyR opening that fails to trigger other RyRs in the same couplon), to primary sparks, to macrosparks (primary spark plus several secondary sparks), to abortive waves (primary spark plus many secondary sparks), to full waves (primary spark plus very many secondary sparks), and even to self-sustaining reentrant rotors (all secondary sparks)\textsuperscript{21}. Key properties of the network arise naturally and play critical roles in the regulation of contractile force. These include graded Ca release\textsuperscript{25} with variable ECC gain and a steep dependence of Ca release on SR Ca load\textsuperscript{15}. Arrhythmogenic patterns of Ca release, including Ca waves and Ca alternans, can also arise.

1.4 The dynamical emergence of calcium-mediated arrhythmias from the CRU network

1.4.1 Statement of the problem & Overview of the dissertation

In this body of work, we uncover and investigate the dynamics-based mechanisms by which the broad spectrum of spontaneous Ca release behaviors emerge from the CRU network, and aim to understand the means by which these behaviors promote arrhythmias across the subcellular, cellular, and tissue scales of integration in the heart. As such, there are three main aspects of cardiac arrhythmias we aim to address. The first is to understand the key relationships among properties of the CRU network itself that give rise to various patterns of
subcellular Ca release (Chapters 2 & 3). The second is to comprehend how these spontaneous Ca release behaviors shape and influence EADs and DADs to trigger arrhythmias and also increase tissue substrate vulnerability (Chapters 4 & 5). The third is to elucidate what will be referred to here as the multiscale problem, which is the basis by which properties or functions of one or a group of biological components at one scale of biological organization are able to traverse scales and assert their influence on other scales (Chapter 5). Overall, this body of work explores an intriguing context in which the deceptively complex behaviors that emerge from the few and simple rules underlying deterministic processes in the heart intertwine with the unpredictable nature of stochastic processes to generate cardiac arrhythmias.

1.4.2 Methodology

The investigative endeavors utilized in this body of work are a multidisciplinary effort combining largely experimental single-cell patch clamp electrophysiology and live Ca fluorescence imaging techniques (Figs. 1.5, 1.6) with computational modeling and theoretical analysis (Fig. 1.7). This

![Simultaneous CCD-based Calcium Imaging of Current-Clamped Ventricular Myocytes](image)

Figure 1.5. Simultaneous experimental patch clamp electrophysiology and Ca fluorescence imaging. Hearts from young adult New Zealand white male rabbits (or from young adult C57Bl mice) were excised by thoracotomy and retrogradely perfused at 37 °C in Langendorff fashion. Individual cardiac myocytes were isolated from the tissue by enzymatic digestion followed by gentle mechanical dissociation. Intracellular Ca activity was recorded in myocytes loaded with the Ca-sensitive dye, Fluo-4, using a charge-coupled device (CCD)-based camera (~100 frames/s, 128x128 pixels). Concurrent electrophysiologial data were acquired using single-cell patch clamp electrophysiology techniques.
multidisciplinary effort is synergistic in form such that the biological heart will serve as the basis for conducting nonlinear dynamics analysis to derive and establish theories for the dynamical processes underlying arrhythmias. The theoretical predictions, in turn, will serve as the basis for gaining new insights into the nature of arrhythmias and for designing therapeutic strategies. Furthermore, theories will be validated experimentally, following precedent set forth by the tradition of physics, as well as through virtual computational simulations for experiments that are not technically feasible.

Figure 1.6. Confocal imaging of Ca fluorescence activity in permeabilized myocytes.
A. Isolated myocytes were permeabilized with saponin and superfused with mock internal solution with Ca-sensitive dye (Fluo-4) in order to control intracellular Ca levels and investigate subcellular Ca release activity without the effects of Ca regulation by surface membrane proteins.
B. Laser scanning confocal microscopy techniques were used to record subcellular Ca activity in high spatial and temporal resolution. Ca release activity was represented as space-time plots of repeated confocal laser line scans on a single linear region of the myocyte over time.
Figure 1.7. Computer modeling and simulations.

A. Biological systems such as subcellular Ca cycling molecular components within myocytes can be represented in silico through computer modeling. A CRU network model of 20,000 diffusively coupled CRUs was used in several studies in this body of work.

B. The model was capable of generating the Ca signaling hierarchy of Ca release behaviors including sparks, macrosparks, and waves in simulations.

C. The results of the model system shared much qualitative similarity with Ca release behaviors recorded from real myocytes.
2

Criticality in the calcium spark to wave transition
From within the CRU network, key properties arise naturally and play critical roles in the regulation of contractile force. These include graded Ca release\(^{25}\) with variable ECC gain and a steep dependence of Ca release on SR Ca load\(^{15}\). Arrhythmogenic behaviors, including intracellular Ca waves and alternans, can also arise. To explore the relationship between the properties of CRUs and those that emerge at the network level, our group developed a conceptual model representing CRUs abstractly as diffusively-coupled excitable elements\(^{25}\). Using this model, we identified three critical parameters among CRUs and the CRU network that provide a unifying general theory linking intracellular Ca cycling dynamics to emergent phenomena. We call these properties the “3R’s,” and they represent the \_Randomness\_ of Ca spark activation, \_Refractoriness\_ of a CRU after firing a spark, and \_Recruitment\_ of Ca sparks in adjacent CRUs induced by Ca sparks\(^{23, 26}\). Based on our theoretical analysis using the conceptual model, we postulated our ‘3R theory,’ which asserts that the range of spontaneous subcellular Ca release behaviors such as Ca waves are interrelated, collective behaviors of the CRU network that arise dynamically from interactions among the 3R’s (Fig. 1.4).

The 3R theory is consistent with the typical nonlinear dynamics view of deterministic systems in that system behaviors are qualitatively new properties that emerge due to nonlinear interactions among its units. Yet, what makes the CRU network context particularly unique is that while the behavior of the system in the form of propagating Ca waves is deterministic in nature, the individual behavior of CRUs is inherently stochastic. The simple illustration that follows demonstrates how the transition from stochastically firing Ca sparks to propagating Ca waves is more than a randomly occurring process. First, consider that the initiation of Ca waves requires many Ca sparks to fire near-simultaneously and in near proximity. If CRUs independently release Ca sparks on a purely random basis, the probability of observing larger clusters of Ca sparks would become exceedingly and exponentially low. According to calculations of Ca spark frequencies (0.06 sparks/sec/CRU) based on experimental measures\(^{22}\), the likelihood of observing a Ca wave would be on the order of days. However, Ca waves are
experimentally observed every 6-10 seconds\textsuperscript{22}, which means that the probability of observing larger clusters of Ca sparks is much more frequent than predicted by a random process, which can be represented with an exponential distribution.

In this chapter, we investigate the mechanistic process that underlies the transition from randomly occurring Ca sparks to propagating Ca waves at the subcellular level in cardiac myocytes using a combined experimental and computational approach. Our findings indicate that the distribution of spark cluster sizes near the critical transition state from sparks to waves follows a power-law distribution, implicating the role of a universal process of criticality in the emergence of arrhythmogenic Ca waves (Fig. 2.1).

**Figure 2.0. Criticality in physical and biological systems.** The rice pile model of avalanches is the classic model of criticality in physical systems\textsuperscript{27}. In this model, rice grains are allowed to fall randomly in space and time into a pile. Rice grain avalanche sizes tend to increase as the steepness of the slope of the pile increases until a critical slope at which avalanches of all sizes form. The transition from Ca sparks to the formation of spark clusters and waves follows an analogous process. Each system exhibits scale-invariant power-law distributions in the sizes of avalanches or Ca spark cluster sizes, respectively, suggesting that Ca wave formation in cardiac myocytes is also due to a process of criticality.
Criticality in Intracellular Calcium Signaling in Cardiac Myocytes

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ABSTRACT  Calcium (Ca) is a ubiquitous second messenger that regulates many biological functions. The elementary events of local Ca signaling are Ca sparks, which occur randomly in time and space, and integrate to produce global signaling events such as intra- and intercellular Ca waves and whole-cell Ca oscillations. Despite extensive experimental characterization in many systems, the transition from local random to global synchronous events is still poorly understood. Here we show that criticality, a ubiquitous dynamical phenomenon in nature, is responsible for the transition from local to global Ca signaling. We demonstrate this first in a computational model of Ca signaling in a cardiac myocyte and then experimentally in mouse ventricular myocytes, complemented by a theoretical agent-based model to delineate the underlying dynamics. We show that the interaction between the Ca release units via Ca-induced Ca release causes self-organization of Ca spark clusters. When the coupling between Ca release units is weak, the cluster-size distribution is exponential. As the interactions become strong, the cluster-size distribution changes to a power-law distribution, which is characteristic of criticality in thermodynamic and complex nonlinear systems, and facilitates the formation and propagation of Ca waves and whole-cell Ca oscillations. Our findings illustrate how criticality is harnessed by a biological cell to regulate Ca signaling via self-organization of random subcellular events into cellular-scale oscillations, and provide a general theoretical framework for the transition from local Ca signaling to global Ca signaling in biological cells.

INTRODUCTION

Calcium (Ca) signaling regulates many biological functions (1–4), including fertilization, gene transcription, cell division, cell death, hormone release, muscle contraction, and heart rhythm and contraction. Although the details of the Ca signaling pathways vary among cell types, the general principles of regulation are universal. Ca is stored in intracellular organelles such as the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR), from which it is released into the cytoplasmic space through opening of Ca-sensitive inositol 1,4,5-trisphosphate receptors (IP₃Rs) or ryanodine receptors (RyRs). The open probability of both IP₃R and RyR increases as cytoplasmic Ca increases, a process called CICR, which is a key positive feedback loop underlying the rich Ca dynamics observed in biological cells (5–13). Ca release from the ER or SR is triggered by Ca entry from the voltage-gated or ligand-gated Ca channels in the cell membrane, or in response to signal transduction, such as G-protein-related signaling pathways. Spontaneous Ca release can also occur under certain conditions, such as Ca overload. A basic unit of Ca signaling is a Ca release event from a single or a few RyRs or IP₃Rs in a CRU called a spark (16), and a discrete Ca release event resulting from the collective openings of many RyRs and IP₃Rs due to the positive feedback mediated by CICR called a spark (17). Ca sparks are considered to be elementary events of Ca signaling, and tend to occur randomly in space and time. A hierarchy of Ca signaling that includes quarks, sparks, spark clusters, and abortive and persistent Ca waves has been observed. It has also been observed in many studies (9–12,18) that the origins of Ca waves vary randomly in time and space. The link between Ca sparks and Ca waves has been documented in many experiments (8–12), but it is not clear why some sparks can initiate waves while the vast majority of sparks do not. It is well known that in an excitable medium, due to the source-sink effect, a group of cells (such as the sinoatrial node in the heart) must be excited synchronously to initiate a wave. By the same reasoning, one would expect that to initiate a Ca wave, a spark cluster of a certain size is needed. However, if a spark cluster forms simply by chance, estimation based on experimental data gives rise to a wave frequency much lower than that observed in experiments (11,12) (see estimation in Supporting Material). Therefore, purely random clustering cannot explain the transition from Ca sparks to Ca waves, and one must establish a mechanistic link between the two to fully understand the mechanisms of Ca signaling in biological cells.

Using computer simulations of an IP₃R cluster array model, Falcke (19) first showed that nucleation from
Ca sparks to Ca waves is responsible for intracellular Ca oscillations. Later studies by Skupin et al. (7,20) and Thurley et al. (21) showed that the whole-cell Ca oscillations are intrinsically stochastic, which is not an oscillatory behavior of a IP$_R$/R cluster but rather an emergent behavior of the IP$_R$/R cluster network of the whole cell. Investigators have also carried out computer simulations in cardiac myocyte models (22–26) to study the self-organization of random RyR openings or sparks into Ca waves and triggered activity in cardiac myocytes. However, despite many modeling and experimental studies on the transitions from Ca sparks to Ca waves and whole-cell oscillations, a general theoretical framework for the transition from Ca sparks to Ca waves is still lacking. In this study, we combined computer simulation and experiments to address the question of how local random Ca signaling events self-organize into global Ca signaling events, such as Ca waves and whole-cell oscillations, in cardiac myocytes. Computer simulations of a mathematical model of Ca signaling in cardiac myocytes were performed in combination with theoretical analyses of an agent-based model of excitable elements, and experimentally validated in Ca imaging experiments in permeabilized mouse ventricular myocytes.

We show that the interaction between CRUs via CICR causes self-organization of Ca spark clusters. When the coupling between CRUs is weak, the cluster-size distribution is exponential. As the interactions become strong, the cluster-size distribution changes to a power-law, which facilitates the formation of large spark clusters and preferable spatial conditions for Ca wave initiation and propagation. A power-law distribution is an indicator that a system is in a critical state, such as the critical phenomena seen in second-order phase transitions in thermodynamics and statistical physics (27,28), and self-organized criticality (SOC) observed in many complex nonlinear systems in nature (29–31). Here we show that criticality is harnessed by a biological cell to regulate the self-organization of random subcellular events into cellular-scale oscillations.

**MATERIALS AND METHODS**

### Ca cycling model of ventricular myocytes

We developed a ventricular myocyte Ca cycling model that was improved from a previous model (32) and is similar to other published models for Ca sparks and waves in cardiac myocytes (22–31). The model is a three-dimensional (3D) CRU network (Fig. 1 A) with the CRUs coupled via Ca diffusion in the myoplasmic (Myo) space and SR. Ca concentrations in the Myo and SR space are described by differential equations. Each CRU contains four compartments (Fig. 1 B): network SR (NSR), junctional SR (jSR), Myo, and dyadic space (DS). The arrows in Fig. 1 B illustrate the L-type Ca channel (LCC) flux ($J_{\text{LCC}}$), RyR release flux ($J_{\text{RyR}}$), SERCA uptake ($J_{\text{SERCA}}$), and diffusive flux ($J_{\text{diff}}$). The governing differential equations for the Ca concentrations in different spaces are as follows:

\[
\beta_a(c_a) \frac{dc_a}{dt} = D_m \nabla^2 c_a + J_a
\]  

\[
\beta_i(c_i) \frac{dc_i}{dt} = D_i \nabla^2 c_i + J_i
\]  

\[
\beta_a(c_a^{(0)}) \frac{dc_a^{(0)}}{dt} = J_{d}^{(0)}
\]  

\[
\beta_i(c_i^{(0)}) \frac{dc_i^{(0)}}{dt} = J_{d}^{(0)}
\]

where \(c_a^{(0)}\) and \(c_i^{(0)}\) are the local Ca concentrations in the Myo and the SR spaces, respectively, and \(c_a^{(0)}\) and \(c_i^{(0)}\) are the Ca concentrations in the L and jSR spaces, respectively. \(\beta_a\), \(\beta_i\), \(\beta_a\), and \(\beta_i\) are Ca buffering functions, which are instantaneous functions of the Ca concentration in each space, following Wagner and Keizer (38). \(J_{\text{dut}}, J_{\text{d}}, J_{i}^{(0)}, \) and \(J_{d}^{(0)}\) are the net Ca flux for each space. \(D_m\) and \(D_i\) are the Ca diffusion constants in the Myo space and the SR space, respectively, and we set them as \(D_m = D_i = 0.3 \mu m^2/\text{ms}\). The detailed mathematical formulations of the buffering constants and the Ca fluxes are based on the model by Rovetti et al. (32) and are described in more detail for the model presented here in our recent publication (39).

Computer simulations were performed on a single NVIDIA Tesla C2050 high-performance Fermi-based graphics processing unit (GPU; http://www.nvidia.com). We wrote the programs in C++ using the CUDA API, and used the GNU C++ compiler version 4.4.3 and NVIDIA CUDA version 4.0. Details regarding the numerical algorithms and implementation of GPU computing can be found in our recent publication (39).
Theoretical model

The CRUs were modeled by a three-state cycle (excitable → excited → refractory → excitable; see Fig. 6 A) modified from a previous model (40). We used 100 × 20 × 10 CRUs. The CRUs were coupled to their six nearest neighbors. The transition from the excitable state to the excited state of a CRU in the network was simulated stochastically with the transition rate to be $J = a + \gamma^2$, where $x$ is the number of its six nearest neighboring CRUs in the excited state, $a$ is the rate constant for spontaneous teting, and $\gamma$ is the coupling strength. The spark duration (or the duration of the excited state) is fixed at 60 ms and the refractory period (the time in the refractory state) is fixed at 300 ms. For the results shown in Fig. 6 E, the variable $x$ in the transition rate equation is the number of its 10 (including the nearest diagonal elements) nearest neighboring CRUs in the excited state.

Myocyte preparation

Methods developed by Mitra and Morad (41) to enzymatically isolate cardiac ventricular myocytes were modified for mice. Briefly, C57Bl/6 mice (6–8 weeks old) were injected intraperitoneally with 800 IU heparin (5000 U/ml) 20–30 min before they were anesthetized and then killed with isoflurane (Phoenix Pharmaceuticals). Hearts were quickly excised by thoracotomy and retrogradely perfused on a Langendorff apparatus maintained at 37 °C. The enzyme digestion step consisted of perfusing Tyrode’s solution containing 1 mg/ml collagenase (Type II, 300 U/mg; Worthington) and 2.8 mg/ml protease (Type XIV , Sigma) for 13–15 min. Myocytes were dissociated from digested ventricles by gentle mechanical dissociation and used within 4–6 h. The modified enzyme digestion step consisted of perfusing Tyrode’s solution containing 1 mg/ml collagenase (Type II, 500 U/ml; Worthington) and 2.8 mg/ml protease (Type XIV, 5.5 U/mg; Sigma) for 13–15 min. Myocytes were dissociated from digested ventricles by gentle mechanical dissociation and used within 4–6 h. The modified Tyrode’s solution contained (in mM) 100 potassium aspartate, 20 KCl, 10 HEPES, 0.25–1 EGTA, and 0.03 Fluo-4 (Invitrogen), and 8% w/v dextran (molecular weight 10,000). Myocytes were permeabilized with saponin (0.005% w/v) in a mock internal solution composed of (in mM) 100 potassium aspartate, 20 KCl, 10 HEPES, 0.25–1 EGTA, and 0.75 MgCl$_2$, pH 7.2 (KOH). Permeabilized myocytes were resuspended in a saponin-free mock internal solution composed of (in mM) 100 potassium aspartate, 20 KCl, 5 K$_2$HPO$_4$, 5 MgATP, 10 phosphocreatine, 5 U/ml creatine phosphokinase, 10 HEPES, 0.25–1 EGTA, 5 × 10$^{-10}$–5 × 10$^{-6}$ CaCl$_2$, (free), 1 MgCl$_2$ (free), 0.03 Fluoro-4 (Invitrogen), and 8% w/v dextran (molecular weight ≈40,000, presents osmostic swelling (13)), pH 7.2 (KOH). Free Ca concentration and Mg concentration were calculated with the use of WebMaxC Extended (maxchelator.stanford.edu). Myocytes were obtained from Sigma unless indicated otherwise. All procedures complied with the policies of the Animal Research Committee of the University of California, Los Angeles.

Myocyte permeabilization

Myocyte membranes were permeabilized for 30–60 s with saponin (0.005% w/v) in a mock internal solution composed of (in mM) 100 potassium aspartate, 20 KCl, 10 HEPES, 0.25–1 EGTA, and 0.75 MgCl$_2$, pH 7.2 (KOH). Permeabilized myocytes were resuspended in a saponin-free mock internal solution composed of (in mM) 100 potassium aspartate, 20 KCl, 5 K$_2$HPO$_4$, 5 MgATP, 10 phosphocreatine, 5 U/ml creatine phosphokinase, 10 HEPES, 0.25–1 EGTA, 5 × 10$^{-10}$–5 × 10$^{-6}$ CaCl$_2$, (free), 1 MgCl$_2$ (free), 0.03 Fluoro-4 (Invitrogen), and 8% w/v dextran (molecular weight ≈40,000, presents osmostic swelling (13)), pH 7.2 (KOH). Free Ca concentration and Mg concentration were calculated with the use of WebMaxC Extended (maxchelator.stanford.edu). All experiments were performed at room temperature. Procedures were slightly modified from a previous study (42).

Ca imaging

We imaged spontaneous Ca activity in permeabilized myocytes using a Zeiss PASCAL 5 laser scanning confocal system (Carl Zeiss) on a Zeiss Axiosvert 100 LSM inverted microscope fitted with a 63X objective (Zeiss C-Apochromat 63/1.2 W Corr). The Ca indicator dye Fluo-4 was excited at 488 nm wavelength with an argon/krypton laser with intensity attenuated to 1−3%. Emission wavelengths > 510 nm were detected by the photomultiplier. Fluorescence intensity space-time recordings were acquired in the line scan mode (1.92 mlslice, 2048 lines/recording) along the longitudinal axis of the myocyte and digitized into 1024 × 2048-pixel images (12-bit) line scan with nominal pixel dimensions of 0.08–0.13 μm.

Spark detection and analysis

The SparkMaster (43) plugin for ImageJ software (44) was used to detect and analyze Ca sparks. The analysis parameters were as follows (see Picht et al. (43) for definitions): scanning speed, 520.8 lines/s; pixel size, 0.08–0.13 μm; spark threshold criteria, 3.8 (3.2 for high EGTA); background, 550–1300; and analysis intervals, 5. Due to Sparkmaster’s limitations in detecting sparks within high-noise backgrounds, we implemented a custom absolute-threshold algorithm in recordings with Ca waves, as suggested previously (45). We measured Ca spark cluster sizes (spatial widths) in line scan using a custom algorithm that defines a Ca spark cluster as Ca sparks separated by less than the single spark average full width in space and the single spark average full duration in time. Cluster detection in computer simulations was similar. The onset of a spark was defined as the time at which the dyadic space Ca increased to 400% of baseline, and the termination of a spark was defined as the time at which the dyadic space Ca fell back to within 25% of baseline. A spark cluster was defined as any sparks that are nearest neighbors in space and separated in time by an interval less than or equal to the average single spark duration.

RESULTS

We first performed computer simulations using the spatially distributed Ca cycling model at different Ca loads. Fig. 2 A shows snapshots of free cytoplasmic Ca concentration at four different Ca loads achieved by increasing the extracellular free Ca concentration in the myocyte model (see Movie S1, Movie S2, Movie S3, and Movie S4 for spatiotemporal dynamics). At low Ca loads, the cell predominately exhibits single Ca sparks. As the Ca load increases, more and more sparks occur in clusters. At high Ca loads, the self-organized large clusters propagate as abortive or persistent waves. The average cytoplasmic Ca of the whole Ca imaging events in permeabilized mouse ventricular myocytes superfused with the fluorescent Ca indicator Fluo-4 and imaged with confocal microscopy (see Materials and Methods).

Fig. 3 A shows line scans (space-time plots) of the cytoplasmic free Ca as the free Ca in the bathing solution was increased, demonstrating the same hierarchical Ca dynamics transitioning from sparks to waves observed in the simulations. Fig. 3 B shows spatially averaged Ca fluorescence versus time for the corresponding Ca loads, showing the transition from small fluctuations to large fluctuations and more periodic oscillations. The cluster-size

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distribution obtained using the line scan data changes from exponential to power-law (Fig. 3 C), agreeing with the computer simulation results shown in Fig. 2. For a more direct comparison, we plotted line scans (Fig. 4 A) from the simulations shown in Fig. 2 and used them to recalculate the cluster-size distributions (Fig. 4 B), which produced closer agreement with the experimental data. Note that the cluster-size distributions of the line scans exhibit a stronger exponential component compared with the 3D data (compare Fig. 4 B with Fig. 2 C), suggesting that the cluster distributions in the experimental data would exhibit an even stronger power-law component if one could obtain accurate 3D recordings in a real cell.

Buffering cytoplasmic Ca more tightly by increasing the EGTA concentration (while maintaining the same free Ca concentration) is predicted to weaken the CRU interaction, and has been shown experimentally to suppress Ca waves without altering spark frequency (10,13). Increasing the Ca buffering in the computer model also suppressed Ca waves and changed the power-law distribution to an exponential distribution (Fig. 5 A). These data indicate that coupling between CRUs plays an important role in criticality. This prediction was validated experimentally, as shown in Fig. 5 B. At 400 nM Ca concentration and 0.5 mM EGTA, abortive and persistent Ca waves with a power-law cluster distribution were typically observed (Fig. 3 C). When EGTA was increased to 1 mM while the same free Ca concentration was maintained at 400 nM, Ca waves were suppressed and the cluster distribution became exponential (Fig. 5 B).

To characterize this process from a more theoretical perspective, we constructed a simplified model based on our previous work (40) (see Supporting Material) in which the intrinsic spark rate ($\alpha$) and the coupling strength ($\gamma$) between CRUs were formulated as independently adjustable parameters. CRUs were represented by stochastic agents modeled as three-state cycles (Fig. 6 A) and coupled in a 3D array with nearest-neighbor coupling. When the CRUs were uncoupled ($\gamma = 0$), we observed exponential distributions of cluster size for small $\alpha$-values. As $\alpha$ increased to a critical value ($\alpha = 0.008$), the cluster-size distribution became a power-law (Fig. 6 B), which agrees with traditional percolation theory (Supporting Material) (46,47). However, the value of $\alpha$ estimated from our experimental data and simulations, as well as previous experiments (11) (Supporting Material), is much lower than that required for a power-law distribution in percolation theory. When we used $\alpha = 0.0001$ and coupled the CRUs with a nonzero $\gamma$,
however, the cluster-size distribution changed from an exponential distribution to a power-law distribution at large $g$-values (Fig. 6C), indicating the importance of the CRU-CRU interaction in promoting criticality.

For a wave to self-form in an excitable medium, a sufficiently large cluster needs to form to overcome the source-sink effect for propagation (48). This same effect was also demonstrated in experiments for Ca wave propagation (49). If such a large cluster were to form simply by chance, the wave frequency would be significantly lower than was observed in the experiments (see Supporting Material for details) (11,12). According to the results shown in Fig. 6B and C, the coupling of CRUs plays an important role in forming such large clusters. Of even more importance, coupling enhances the spatial conditions required for a large cluster to propagate as a wave, because the clusters that form via the self-organizing process have much more excitable space for propagation compared with clusters that form randomly due to a high spark probability (Fig. 6D).

One discrepancy between the agent-based theoretical model and the detailed model is that the power-law exponent is smaller in the detailed model. We reason that in the detailed model, free Ca diffusion in the cytoplasmic space extends CRU coupling beyond the nearest neighbors, whereas the theoretical model exhibits only nearest-neighbor coupling. When we increased the coupling in the theoretical model to include the nearest diagonal neighbors, we observed a larger exponent (Fig. 6E), which is more consistent with the simulation results shown in Fig. 2C.

**DISCUSSION**

A power-law distribution has been considered as a hallmark of criticality (27–31). In physics, criticality occurs in second-order phase transitions of thermodynamic equilibrium systems (27,28), such as the paramagnetic to ferromagnetic transition at the critical (or Curie) temperature. Bak et al. (29) put forward a concept called SOC to explain widely observed power-law distributions in complex nonlinear systems in nature (30,31), in which the system self-organizes into a critical state without the requirement of fine-tuning parameters, in contrast to traditional critical phenomena. SOC has been demonstrated in random excitable systems (50,51) and in a cellular automaton model of Ca signaling (52), as well as in many other systems in nature (e.g., avalanches, earthquakes, forest fires, evolution, ecosystems, and neuronal activity) (30,31,53–57) through...
the observation of power-law distribution of spatial clustering and frequency of temporal fluctuations. In the intracellular Ca signaling systems shown in this study, we observed a power-law distribution before and after Ca waves, occurring over a wide parameter range. On the basis of previous studies of excitable systems (50–52) and our own observations in this study, we believe that SOC is also likely to be the best descriptor of Ca signaling dynamics at the subcellular level, as explained below. In the intracellular Ca signaling system, the Ca load must be raised to a high enough level, such that the individual CRUs are sufficiently excitable and properly coupled, to bring the system to the critical state at which a power-law cluster distribution exists and Ca waves start to occur. When the Ca load is beyond the critical value, Ca waves occur more frequently. As a wave propagates through the system, it empties the SR, and the system is brought out of the critical regime. However, as the SR refills and the RyRs recover, the CRUs regain their excitability and the system self-organizes back into the critical state at which a power-law is achieved. In this scenario, the power-law distribution is observed over a wide range of Ca loadings in our system, in agreement with the SOC scenario.

Compared with an exponential cluster-size distribution, a power-law distribution gives rise to a much higher probability for large spark cluster formation. Once a cluster reaches a critical size, it becomes capable of initiating a wave and giving rise to a whole-cell Ca signal, depending on the status of the surrounding CRUs. As shown in our theoretical model, a low spontaneous spark probability with strong coupling not only causes the formation of large clusters but also sets the permissive spatial conditions for wave propagation. After a wave originating from one location propagates through the cell, it resets the system, and self-organization of spark clusters begins anew after the CRUs recover (see Movie S3 and Movie S4). A new large cluster may form in another location and initiate a new wave, which agrees with experimental observations that the origins of Ca waves vary randomly in space and time (10–12,18). Due to the randomness in cluster formation in time and space, the whole-cell Ca signal exhibits an irregular burst-like behavior, in agreement with experimental observations in many cell types that Ca oscillations are irregular (7,18,20). However, as the coupling strength increases, the periodicity of the oscillations increases due to more synchronous firing of the individual elements.

Ca waves in ventricular myocytes are linked to cardiac arrhythmias by causing delayed afterdepolarizations (58,59) and early afterdepolarizations (60), which tend to occur irregularly (18,61). Our finding that Ca waves start to form when the system reaches a critical state indicates

FIGURE 4 Cluster-size distribution obtained using line scans from the computer simulations. (A) Space-time plots of Ca concentration along a line through the ventricular myocyte model (see corresponding simulations in Fig. 2). (B) Cluster-size distributions obtained using the line scan data from the corresponding simulations. Symbols are data from simulations and lines are reference lines. The line in the leftmost panel in B is a pure exponential.
that the irregularity of delayed afterdepolarizations is amplified from microscopic random ion channel fluctuations, which may provide a subcellular origin of the unpredictability of cardiac arrhythmias and sudden cardiac death (62). In addition, our finding that a strong coupling between CRUs gives rise to much higher probabilities of large spark clusters at low spark rates than would be expected if it were governed by pure chance. In the transition from random sparks to Ca waves, the whole-cell Ca signal changes from small random fluctuations to random bursts, and then to more periodic oscillations. These oscillations arise from the self-organized wave activities and do not require the preexistence of any pacemaking sites in the cell, in agreement with the recent observation that whole-cell Ca oscillations are emergent phenomena of the coupled CRU network (21). Therefore, our study provides a general theoretical and mechanistic framework for the transition from random Ca sparks to Ca waves and whole-cell Ca oscillations, not only in cardiac myocytes but also in other biological cells.

LIMITATIONS

This study has several limitations. Due to the limited system size imposed by a real myocyte, the power-law distribution does not extend far beyond one order of magnitude. However, if we repeat the simulations in a larger system, we observe a power-law distribution that persists over a larger range of cluster sizes (see Fig. S1 for a cluster-size distribution from a larger system of the agent-based
We used the RyR model developed by Stern et al. (36), which exhibits cytosolic Ca-dependent inactivation but lacks the detailed fine structure in a cardiac myocyte that models. The model is a network of coupled CRUs, which may play important roles in intracellular Ca signaling.

In real cardiac myocytes, RyR cluster distribution is somewhat heterogeneous rather than uniform (72), which may also affect the Ca signaling dynamics. Nevertheless, Ca signaling has been shown in many cell types and conditions to exhibit universal behaviors (2), indicating that the universal properties, which is the main goal of this study.

SUPPORTING MATERIAL

Supplemental materials, references, a figure, and four movies are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(12)00520-6.

We thank Dr. Enno de Lange for help with the GPU computing.

This work was supported by National Institutes of Health grants P01 HL078931, R01 HL103622, and T32 GM065823 (C.K.), a postdoctoral fellowship award (M.N.) and a predoctoral fellowship award (C.K.) from 2HL078931, R01 HL103622, and T32 GM065823 (C.K.), a postdoctoral fellowship award (C.K.), and Kawata endowments.

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Criticality in Intracellular Calcium Signaling in Cardiac Myocytes

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Supplemental Materials

Estimation of wave rates based on random spark clustering

The experimental study by Cheng et al (1) observed 3 sparks per sec per line scan, and 1 to 6 waves per min. Assuming that there are 100 CRUs in a line scan, the spark rate of a CRU is $3/100$ per CRU per sec and the total spark rate in a cell containing 20,000 CRUs is: $20,000 \times 3/100 = 600$ sparks per sec = 36,000 sparks per min. Therefore, assuming that the experiment captured all waves, the wave to spark ratio ranges from 1:36,000 to 1:6,000. Using this observed spark and wave data, we can roughly estimate the probability of observing a wave under the assumption that the clustering is purely by chance. In one dimension, if the spark probability of a CRU is $p$, then the probability of observing a cluster of size $s$ due to pure chance is $p^s(1-p)^{2-s}$. However, in higher dimensions this calculation becomes more complicated, and an exact solution cannot be written down. Here we use a crude upper bound to estimate the probability of observing a wave under the assumption that the clustering is purely by chance, which does not pose a problem since this can only result in an overestimation of the wave rate.

i. Assume that the spark duration is 60 ms, thus, two neighboring sparks occurring within 60 ms are considered as a cluster. The rate for at least two neighboring CRUs to fire within 60 ms is $(3/100 \times 60/1000)^2$. Since there are roughly 20,000 CRUs in a cell, 20,000 serves as an upper bound for the number of possible 2-spark clusters in a cell. Therefore, an upper estimate for the number of two-spark clusters is: $20,000 \times (3/100 \times 60/1000)^2$ per sec = 0.0648 per sec = 3.9 per min.

ii. For three neighboring sparks occurring within 120 ms as a cluster, the rate is: $20,000 \times (3/50 \times 120/1000)^3$ per sec = 0.0075 per sec = 0.4479 per min.

iii. For four neighboring sparks occurring in 180 ms as a cluster, the rate is: $20,000 \times (3/50 \times 180/1000)^4$ per sec = 0.00027210 per sec = 0.00012383 per sec = 2.7 per day.

In both our experiments and simulations, we observed that a cluster much larger than 5 sparks is needed to overcome the source-sink effect to propagate as a wave. Therefore, large enough clusters forming purely by chance cannot account for the high wave rate seen in experiments, e.g., Cheng et al (1) observed 3 sparks per sec per line scan and 1-6 waves per min, Wier et al (2) observed 12 sparks per sec per line scan and 1 wave every 2 sec. Our experiments show similar spark and wave rates with 10-50 sparks per sec per line scan and 1 wave every 1-3 sec.

Percolation threshold in an array of stochastic agents

Here we employ a simplified model consisting of an array of stochastic elements. Each element of the array is modeled by a three-state cycle (Fig.4a in the main text), similar to the model used by Cui et al (3):

excitable $\Rightarrow$ excited $\Rightarrow$ refractory $\Rightarrow$ excitable. \hspace{1cm} (1)

An element with spatial coordinates $(i, j, k)$ remains in the refractory state for a fixed dwell time $\tau_{\text{refrac}}$, after which it transitions to the excitable state. It then transitions to the excited state with Markov transition rate

$\lambda(i, j, k) = \alpha + \gamma s(i, j, k)^2$, \hspace{1cm} (2)
where $\alpha$ is the constant transition rate corresponding to the intrinsic spark latency, and $\gamma$ governs the strength of the coupling, which depends nonlinearly on $s$, the number of nearest neighbors in the excited state. It remains in the excited state with fixed dwell time $\tau_{\text{dur}}$, the spark duration. We let this network evolve in time and observe the clustering dynamics, where a cluster is defined as any elements of the array which are in the excited state and are nearest neighbors in space and separated in time by an interval less than or equal to the spark duration $\tau_{\text{dur}}$. Based on experimentally observed values, we take $\tau_{\text{refrac}} = 300 \, \text{ms}$, $\tau_{\text{dur}} = 60 \, \text{ms}$, and $\alpha = 0.0001 \, \text{ms}^{-1}$ as our baseline parameter values.

In the uncoupled case, clustering is governed by the intrinsic spark rate through purely random association. Classic percolation theory predicts that for a static array in which elements are randomly “bonded” with probability $p$ there is a critical value of the bond probability for which the system exhibits scale-free power-law clustering. This clustering is characterized by its Fisher exponent; when plotted on a log-log plot the cluster-size distribution is a straight line with slope given by the negative of the Fisher exponent (4). Far below the percolation value the cluster-size distribution is exponential. However, this is a static picture. Our definition of cluster is not static but dynamic, and we are interested in cluster formation as it happens in time. Therefore, in order to apply results from classic percolation theory, we must first relate our dynamic problem to this classic bond percolation problem, we do so below.

Consider the uncoupled system, setting $\gamma = 0$. Without loss of generality, assume that a spark has occurred at time $t = 0$. Then we ask the question: According to our definition of cluster, what is the probability that a neighboring spark will be added to the same cluster, i.e., that the excited element will form a bond with one of its neighbors? According to our definition of cluster, if a neighbor is already in the excited state or it transitions to the excited state in the time interval $\Delta t \leq \tau_{\text{dur}}$, then it will be added to the same cluster as the original spark. For a snapshot of the system in a uniform steady state, the probability of finding an element in the excited state is

$$\frac{\tau_{\text{dur}}}{\tau_{\text{dur}} + \tau_{\text{refrac}} + \tau_{\alpha}}$$

where without coupling ($\gamma = 0$), $\tau_{\alpha}$ is the average wait time for the transition from the excitable to the excited state

$$\tau_{\alpha} = \frac{1}{\alpha}$$

The probability of a not already excited element transitioning into the excited state in a time interval $\Delta t \leq \tau_{\text{refrac}} + \tau_{\alpha}$ is

$$(1 - \frac{\tau_{\text{dur}}}{\tau_{\text{dur}} + \tau_{\text{refrac}} + \tau_{\alpha}})(\frac{\Delta t}{\tau_{\text{dur}} + \tau_{\text{refrac}} + \tau_{\alpha}})$$

where the left multiplier is the probability of not being in the excited state, and the right multiplier is the probability of an excitable or refractory agent transitioning to the excited state during time $\Delta t$. Therefore, the probability of finding an agent in the excited state during a time interval $\Delta t$ is

$$p = \frac{\tau_{\text{dur}}}{\tau_{\text{dur}} + \tau_{\text{refrac}} + \tau_{\alpha}} + (1 - \frac{\tau_{\text{dur}}}{\tau_{\text{dur}} + \tau_{\text{refrac}} + \tau_{\alpha}})(\frac{\Delta t}{\tau_{\text{dur}} + \tau_{\text{refrac}} + \tau_{\alpha}})$$

Since we consider two sparks to be neighbors in time if they are separated by a time interval less than or equal to the spark duration, we set $\Delta t = \tau_{\text{dur}}$ in the above. Then the probability that a newly excited agent forms a bond with a neighboring agent simplifies to

$$p = \frac{2\tau_{\text{dur}}}{\tau_{\text{dur}} + \tau_{\text{refrac}} + \tau_{\alpha}}$$
where we must assume \( \tau_{\text{dur}} = \Delta t \leq \tau_{\text{refrac}} + \tau_a \).

The measured bond percolation threshold for a 3-d cubic lattice with 6 nearest-neighbor coupling is \( p^* = 0.2488126 \). Evaluating the above equation at \( p = p^* \), \( \tau_{\text{refrac}} = 300 \), \( \tau_{\text{dur}} = 60 \) and solving for \( \tau_a \) gives a theoretically predicted value of the spark latency at which the system percolates due to purely random association to be \( \tau_a^* = 122 \) ms which implies

\[
\alpha^* \approx 0.008 \tag{8}
\]

This theoretically predicted value agrees with our simulations (see Figure 4 in main text). Also, at percolation, the cluster-size distribution plotted on a log-log plot is a straight line with slope approximately equal to -2.2. This agrees with the Fisher exponent from classic 3-d percolation \((4, 5)\), which is observed to be approximately 2.186.

An upper bound for the experimentally observed spark rate is on the order of \( r \approx 0.0001 \) (see Section 2.1 of supplement). We can relate the transition rate \( \alpha \) to the experimentally measured spark rate through the formula

\[
\frac{1}{r} = \tau_a + \tau_{\text{refrac}} + \tau_{\text{dur}} \tag{9}
\]

Therefore, we can use the above formula along with our theoretically predicted critical value \( \alpha^* \) to determine at what experimental spark rate one would expect power-law clustering due to purely random association. Substituting \( \alpha = \alpha^*, \tau_{\text{dur}} = 60, \) and \( \tau_{\text{refrac}} = 300 \) and solving for \( r \) gives \( r^* \approx 0.002 \), which is 20 times larger than what is observed experimentally.

Therefore, coupling must play a large role in the self-organization process. For example, with a normal spark latency of \( \alpha = 0.0001 \), power-law clustering is observed at a coupling strength of \( \gamma \approx 0.003 \). Furthermore, the power-law exponent for the cluster-size distribution is approximately -1.7, different from that of classic percolation. Note that in the detailed model, Ca diffusion allows for coupling to extend beyond just the 6 nearest neighbors. In fact, if we allow diagonal neighbors to contribute to the coupling in Equation 2, then the power-law exponent decreases to approximately -2.9, which agrees with the exponent observed in the detailed model.

References

Supplemental Figure

Figure S. Cluster size distribution from the agent-based model composed of 1000x1000 CRUs with $\alpha=0.0001$ and $\gamma=0.008$.

Supplemental Movies:

Movie 1. Ca spark dynamics at $[\text{Ca}]_0=3$ mM
Movie 2. Ca spark and cluster dynamics at $[\text{Ca}]_0=9$ mM
Movie 3. Ca sparks and abortive waves at $[\text{Ca}]_0=10$ mM
Movie 4. Ca waves at $[\text{Ca}]_0=16$ mM
Calcium wave entrainment and pacemaker sites for calcium oscillations
In the previous chapter, we demonstrate that criticality is the underlying theoretical mechanism that governs the transition from randomly occurring Ca sparks to organized and propagating Ca waves, analogous to a second-order phase transition in thermodynamics\textsuperscript{28}. In order for this transition to occur, the CRU network must reach a certain critical state for Ca sparks to self-organize into spark clusters capable of initiating Ca waves. The self-organizing process depends highly upon the degree of Ca-mediated diffusive coupling within the CRU network and gives rise to the probabilistic nature of wave occurrence. When intracellular Ca levels are low and CRU coupling is reduced, the likelihood of forming spark clusters from randomly occurring sparks is low and accounts for long and variable time intervals between Ca waves. When intracellular Ca levels are raised and CRU coupling is enhanced, the likelihood of spark cluster formation increases nonlinearly, reducing and synchronizing the time intervals between waves.

Although the temporal characteristics of subcellular Ca wave emergence observed between real isolated myocytes and computational simulations were consistent in our studies, the spatial patterns pertaining to the sites of Ca wave initiation were inconsistent. As intracellular Ca was raised and wave initiation was induced, Ca waves tended to initiate repeatedly from the same region in real myocytes, while they tended to initiate randomly in space from release to release in the homogeneous construction of the computational model.

This qualitative discrepancy in the spatiotemporal patterns of Ca release in our studies implicates the potentially significant role that heterogeneities have in influencing the emergence of arrhythmogenic Ca waves. These heterogeneities can emerge due to dynamic processes, as explained by the 3R theory, or they can be physical, as observed in structural subcellular remodeling processes in disease contexts such as heart failure\textsuperscript{29}. In this chapter, we investigate the spatiotemporal qualities of Ca release behavior in context of dynamical and structural heterogeneities and derive a universal theory of Ca wave entrainment for the emergence of Ca oscillations and pacemaking sites in the heart.
The emergence of subcellular pacemaker sites for calcium waves and oscillations

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Key points

• Calcium (Ca²⁺) is fundamental to biological cell function, and Ca²⁺ waves generating oscillatory Ca²⁺ signals are widely observed in many cell types.
• Some experimental studies have shown that Ca²⁺ waves initiate from random locations within the cell, while other studies have shown that waves occur repetitively from preferred locations (pacemaker sites).
• In both ventricular myocyte experiments and computer simulations of a heterogeneous model of coupled Ca²⁺ release units (CRUs), we show that Ca²⁺ waves occur randomly in space and time when the Ca²⁺ level is low, but as the Ca²⁺ level increases, waves occur repetitively from the same sites.
• Ca²⁺ waves are self-organized dynamics of the CRU network, and the wave frequency strongly depends on CRU coupling.
• Using these results, we develop a theory for the entrainment of random oscillators, which provides a unified explanation for the experimental and computational observations.

Abstract Calcium (Ca²⁺) waves generating oscillatory Ca²⁺ signals are widely observed in biological cells. Experimental studies have shown that under certain conditions, initiation of Ca²⁺ waves is random in space and time, while under other conditions, waves occur repetitively from preferred locations (pacemaker sites) from which they entrain the whole cell. In this study, we use computer simulations to investigate the self-organization of Ca²⁺ sparks into pacemaker sites generating Ca²⁺ oscillations. In both ventricular myocyte experiments and computer simulations of a heterogeneous Ca²⁺ release unit (CRU) network model, we show that Ca²⁺ waves occur randomly in space and time when the Ca²⁺ level is low, but as the Ca²⁺ level increases, waves occur repetitively from the same sites. Our analysis indicates that this transition to entrainment can be attributed to the fact that random Ca²⁺ sparks self-organize into Ca²⁺ oscillations differently at low and high Ca²⁺ levels. At low Ca²⁺, the whole cell Ca²⁺ oscillation frequency of the coupled CRU system is much slower than that of an isolated single CRU. Compared to a single CRU, the distribution of interspike intervals (ISIs) of the coupled CRU network exhibits a greater variation, and its ISI distribution is asymmetric with respect to the peak, exhibiting a fat tail. At high Ca²⁺, however, the coupled CRU network has a faster frequency and lesser ISI variation compared to an individual CRU. The ISI distribution of the coupled network no longer exhibits a fat tail and is well-approximated by a Gaussian distribution. This same Ca²⁺ oscillation behaviour can also be achieved by varying the number of ryanodine receptors per CRU or the distance between CRUs. Using these results, we develop a theory for the entrainment of random oscillators which provides a unified explanation for the experimental observations underlying the emergence of pacemaker sites and Ca²⁺ oscillations.
Introduction

Calcium (Ca\(^{2+}\)) signalling is fundamental to biological function (Berridge et al. 2000). In the heart, Ca\(^{2+}\) plays crucial roles in pacemaker function underlying the normal heart rhythm (Lakatta et al. 2010) and excitation–contraction coupling (Bers, 2002), while dysfunction in intracellular Ca\(^{2+}\) signalling can promote heart failure (Anderson et al. 2011) and cardiac arrhythmias (ter Keurs & Boyden, 2007). Experimental studies have demonstrated a universal hierarchy of Ca\(^{2+}\) signalling dynamics in cardiac myocytes as well as in many other cell types (Marchant et al. 1999; Berridge et al. 2000; Marchant & Parker, 2001; Cheng & Lederer, 2008; Nivala et al. 2012b), ranging from Ca\(^{2+}\) quarks, sparks, and macrosparks to abortive and finally full waves. This hierarchy can be demonstrated by altering certain parameters, such as extracellular Ca\(^{2+}\) concentration (Capogrossi & Lakatta, 1985; Capogrossi et al. 1986; Cheng et al. 1996) or inositol 1,4,5-trisphosphate (IP\(_3\)) concentration (Marchant et al. 1999; Marchant & Parker, 2001) in the cell. Specifically, at low Ca\(^{2+}\) or IP\(_3\) concentrations, cells predominantly exhibit random single Ca\(^{2+}\) sparks with only small fluctuations in the average cytosolic Ca\(^{2+}\) of the whole cell. As the Ca\(^{2+}\) or IP\(_3\) concentration increases, spark clusters and waves occur, which result in spikes of the whole-cell Ca\(^{2+}\) concentration, with randomly irregular interspike intervals (ISIs). As the Ca\(^{2+}\) or IP\(_3\) concentration increases further, the Ca\(^{2+}\) waves and whole-cell Ca\(^{2+}\) spikes become more periodic in time.

Computer modelling studies using stochastic simulations of Ca\(^{2+}\) release unit (CRU) networks coupled via Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR; Falcke, 2003; Shuai & Jung, 2003; Izu et al. 2006; Skupin et al. 2010b; Nivala et al. 2012b) have shown that the initiation of a Ca\(^{2+}\) wave is not due to a single spark but requires the formation of a locally self-organized cluster of nearby sparks large enough to propagate non-decrementally by CICR. Recently, we showed that the theory of criticality governs the transition from sparks to waves (Nivala et al. 2012b), similar to a second-order phase transition in thermodynamic systems (Stanley, 1999). In other words, as the interaction between CRUs via CICR becomes stronger, the spark cluster-size distribution changes from an exponential distribution to a power-law distribution, characteristic of criticality. Long-range correlations, which occur during criticality, facilitate the formation and propagation of Ca\(^{2+}\) waves. Theoretically, when the distribution of CRUs is uniform inside a cell, the wave initiation sites should occur randomly and uniformly in space, as demonstrated recently in simulations of a homogeneous CRU network (Nivala et al. 2012b). This pattern has been observed in many experimental studies (Cheng et al. 1996; Wier et al. 1997; Marchant & Parker, 2001; Wasserstrom et al. 2010). However, in other experimental studies, waves have also been observed to originate repetitively from the same locations in a cell (Rooney et al. 1990; Kasai et al. 1993; Thorn et al. 1993; Simpson et al. 1997). The latter is believed to be caused by heterogeneities in the CRU network, such as localized higher concentrations of ryanodine receptors (RyRs) or IP\(_3\) receptors in so-called ‘pacemaker’ regions of a cell. In our own experiments in ventricular myocytes (data shown in Fig. 3 of our previous publication (Nivala et al. 2012b) and Fig. 1), we have observed both patterns occurring in the same cell: at Ca\(^{2+}\) levels near the spark-to-wave transition, waves occur irregularly and originate randomly from different sites, while at higher Ca\(^{2+}\) levels, waves become periodic and tend to originate from preferred locations, acting as pacemaker regions which entrain the whole cell. The question arises, therefore, how can both patterns occur in the same cell? That is, why, at low Ca\(^{2+}\) levels, do Ca\(^{2+}\) waves arise randomly and irregularly from multiple sites, while at high Ca\(^{2+}\) levels, specific pacemaker regions emerge as the predominant source of Ca\(^{2+}\) waves generating more periodic Ca\(^{2+}\) oscillations? Here we show that this behaviour is a natural consequence of self-organization and random entrainment in a heterogeneous system.

Methods

Cardiac myocyte experiments

The experimental protocol in this study was approved by the UCLA Animal Research Committee and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

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Ventricular myocyte isolations were performed as previously described (Nivala et al. 2012b). Briefly, male C57BL/6 mice of 68 weeks of age were injected intraperitoneally with 800 ml heparin (5000 U/ml) 20–30 min before anesthetization and euthanization with isoflurane (Phoenix Pharmaceuticals). Hearts were quickly excised by thoracotomy and retrogradely perfused on a Langendorff apparatus maintained at 37 C. The enzyme digestion step consisted of perfusing Tyrode’s solution containing 1 mg/ml collagenase (Type II, 300 U/mg; Worthington) and 2.8 mg/ml protease (Type XIV, Sigma) within 46 h. Myocyte membranes were permeabilized for 13–15 min. Myocytes were dissociated from digested ventricles by gentle mechanical dissociation and used within 46 h. Myocyte membranes were permeabilized for 30–60 s with saponin (0.005% w/v) in nominally Ca2+-free Tyrode’s solution buffered with 0.25 mM EGTA and 0.03 mM Fluo-4 fluorescent Ca2+ dye (Invitrogen), using WebMaxC Extended software (maxchelator.stanford.edu) to calculate free [Ca2+]. Ca2+ imaging was performed using laser confocal microscopy (Zeiss PASCAL 5 system) as described previously (Nivala et al. 2012b). Fluorescence intensity space–time recordings were acquired in the linescan mode (100 µm line–1, 1.92 ms line–1, 2604 or 5208 lines per recording corresponding to 5 or 10 s long recordings, respectively).

**Experimental data analysis**

Experimental linescan recordings were analysed using ImageJ software, and statistical analyses were performed using custom Python code (courtesy of Dr. Guillaume Calmettes). The inter-wave initiation site distances (ISDs) and ISIs (see Fig. 1A) were measured only from consecutive Ca2+ waves that each propagated at least 30 µm. Since the ISD and ISI distributions did not meet conditions for normality, the median was selected as the least biased descriptor for all distributions. Ca2+ wave periodicity was described using ISI variance (σ(ISI)−) modified for non-normal distributions and defined as (σ(ISI1 − ISImedian))/(N − 1). In this formula, the median was used in place of the mean as a more appropriate descriptor of non-normal distributions. The absolute value function was used in place of the squaring function, which tends to bias the standard variance towards larger deviations from the mean. Smaller values of σ(ISI−) reflect greater periodicity. To measure the effect size of intracellular Ca2+ levels on wave periodicity, we used the difference in σ(ISI−) between low and high Ca2+ conditions (i.e. σ(ISIhighCa)− σ(ISILowCa)). Negative effect sizes indicate smaller value measures at high Ca2+ relative to the measures at low Ca2+ levels. Confidence intervals (CIs) around observed statistical measures (i.e. median, σ(ISI−) and effect sizes) were generated using computational bootstrap methods (Efron & Tibshirani, 1991; Manly, 1997; Calmettes et al., 2012), which account for smaller and unequal sample sizes and non-normal distributions. Briefly, the statistical measure of interest was calculated from an individual pseudo data sample set generated by randomly resampling, with replacement, values from an actual data sample set (e.g. ISP(highCa)). This process was repeated 100,000 times to generate a distribution of 100,000 estimates of the variability of the statistical measure due to sampling variations. A 99% CI around an observed statistic was determined as the range of values above the 2500th and below the 97,500th value of the distribution of estimates. Statistical significance at the P < 0.01 level was established by determining whether effect sizes of zero (i.e. no difference between low and high Ca2+ conditions) for a statistical measure fell outside of the 99% CI.

**Computational models and methods**

The basic computational units are CRUs which are coupled via Ca2+ diffusion in myoplasm and sarcoplasmic reticulum (SR) to form a three-dimensional (3-D) cell model (containing 100 × 20 × 10 CRUs). The CRU model was presented in our previous studies (Bovetti et al. 2010; Nivala et al. 2012a,b). Briefly, each CRU contains: a junctional SR (jSR) which is diffusively connected to the network SR (NSR), and a dyadic space (DS), which is diffusively connected to the myoplasm (Myo) space. Extracellular Ca2+ enters the DS via a leak conductance and the voltage-gated L-type Ca2+ channels (LCCs), which were simulated stochastically by a Markov model (Mahajan et al. 2008). Ca2+ is released from the jSR through its associated cluster of RyRs to the DS. The RyRs were simulated stochastically using a Markov model (Stern et al. 1999) in which activation and inactivation of RyRs are regulated by Ca2+ in the DS. Each CRU contains 10 LCCs and 100 RyRs. Ca2+ is either extruded from the cell via the Na+–Ca2+ exchanger or taken back up into the NSR via the SERCA pump. The governing differential equations for the Ca2+ concentration in different spaces are:

\[
\begin{align*}
\beta_m(c_m) \frac{\partial c_m}{\partial t} &= D_m \nabla^2 c_m + f_m, \\
\beta_s(c_s) \frac{\partial c_s}{\partial t} &= D_s \nabla^2 c_s + f_s, \\
\beta_d(c^{(i)}_d) \frac{\partial c^{(i)}_d}{\partial t} &= f^{(i)}_d,
\end{align*}
\]

(1) (2) (3)
\[
\beta_i (c_i(t)) \frac{dc_i(t)}{dt} = J_i(t),
\]
where \(c_m(x,y,z,t)\) and \(c_s(x,y,z,t)\) are the local \(Ca^{2+}\) concentrations in the Myo and the NSR, respectively, and \(c_i^{0}\) and \(c_s^{0}\) are the \(Ca^{2+}\) buffering constants, which are instantaneous functions of the \(Ca^{2+}\) concentrations in the corresponding spaces, following Wagner & Keizer (1994). \(J_m, J_s, J_i^{(0)}\) and \(J_i^{(1)}\) are the net \(Ca^{2+}\) fluxes for each space. \(D_m\) and \(D_s\) are the \(Ca^{2+}\) diffusion constants in the Myo and the NSR, respectively.

The majority of the simulations in the study were performed in quasi-two-dimensional (2-D) models, in which we coupled the CRUs in 2-D arrays of different sizes. The purposes are twofold: to speed up the simulation for statistics and to allow systematic analyses by comparing behaviours of a single uncoupled CRU with those of coupled CRUs. Since the CRUs in our model are identical, reducing the 3-D model to a quasi-2-D model has relatively small quantitative effects (e.g. shifting the wave threshold to a lower \([Ca^{2+}]_o\) value) while preserving qualitative aspects of the system.

In numerical simulations, the NSR and Myo domains are discretized into 3-D spatial grids using a \(0.2 \times 0.2 \times 0.2 \mu m^3\) spatial resolution. The equations are simulated using an operator splitting method by advancing first the diffusion step and then the flux steps using a first-order forward Euler method with a time step of 0.01 ms. The stochastic transitions of LCCs and RyRs are simulated using the Gillespie's stochastic simulation algorithm (Gillespie, 1977), modified to handle time-dependent rates. All computations are performed on an Intel Xeon 2.53 GHz processor using Graphical Processing Unit (GPU) parallel computing with an NVIDIA Tesla C2050. The details of the computational methods were presented previously (Nivala et al. 2012a).

**Results**

**\(Ca^{2+}\) waves transition from random multiple initiation sites to preferred sites as \(Ca^{2+}\) increases in ventricular myocytes**

Permeabilized mouse ventricular myocytes were superfused with the fluorescent \(Ca^{2+}\) indicator Fluo-4 and imaged with confocal microscopy (see Methods). Figure 1A and B compare linescans (space–time plots) of the cytoplasmic \(Ca^{2+}\) fluorescence at low (100 nm) and high (1000 nm) free \(Ca^{2+}\) concentration in the bathing solution. At the low \(Ca^{2+}\) concentration, waves were irregular and initiation sites varied randomly, while at the high \(Ca^{2+}\) concentration in the same myocyte, waves were more periodic and originated more consistently from the same location. Figure 1C shows summary data from multiple cells for the distance between the initiation sites of two consecutive waves, called the inter-wave initiation site distance (ISD). In the case of high \(Ca^{2+}\), more than 80% of the successive wave initiation sites fell in the same 10 \(\mu m\) wide bin, whereas the distribution was much broader for the low \(Ca^{2+}\) case. We also calculated the ISI between waves from the same recordings as used for obtaining Fig. 1C. The ISI was also much more narrowly distributed in the case of high \(Ca^{2+}\) than in the case of low \(Ca^{2+}\) (Fig. 1D). These differences were statistically significant.

For the low \(Ca^{2+}\) condition, ISDs (\(n=29\) wave intervals from 22 recordings in 11 cells) exhibited a much narrower distribution with less skew and a substantially larger proportion of values (ISD\(_{median}=14.44 \mu m\); 99% CI (3.82 \(\mu m\), 29.96 \(\mu m\)). For the high \(Ca^{2+}\) condition (\(n=171\) wave intervals from 16 recordings in 7 cells), ISDs exhibited a much narrower distribution with less skew and a substantially larger proportion of values (ISD\(_{median}=2.76 \mu m\); 99% CI (1.86 \(\mu m\), 4.82 \(\mu m\); Fig. 1C). The two distributions were statistically significantly different (effect size: \(\Delta ISD_{median}=−11.68 \mu m\), 99% CI (−28.06 \(\mu m\), −1.18 \(\mu m\)); \(P<0.01\)). These results indicate multiple sites of wave initiation when \(Ca^{2+}\) is low, but smaller excursions mostly limited to a single or few preferred sites when \(Ca^{2+}\) is high. Corresponding ISIs (Fig. 1D) exhibited a broad distribution (ISI\(_{median}=3.55 s\); 99% CI (3.23 s, 4.13 s)) under the low \(Ca^{2+}\) condition, whereas ISIs exhibited a much narrower distribution and a substantial shift towards smaller values (ISI\(_{median}=0.47 s\); 99% CI (0.42 s, 0.50 s) in the high \(Ca^{2+}\) condition. The two distributions also were statistically significantly different (effect size: \(\Delta ISI_{median}=−3.08 s\); 99% CI (−3.64 s, −2.79 s)). Thus, \(Ca^{2+}\) waves initiate more irregularly at longer intervals when \(Ca^{2+}\) is low, but become more periodic and frequent when \(Ca^{2+}\) is high.

The degree of periodicity was analysed by comparing the average deviation from the median ISI (\(\sigma_{Med}\), see Methods) at low versus high \(Ca^{2+}\). \(\sigma_{Med}\) decreased from 0.34 s (99% CI (0.34 s, 0.74 s)) at low \(Ca^{2+}\) to 0.12 s (99% CI (0.09 s, 0.16 s)) at high \(Ca^{2+}\), which was statistically significant (\(P<0.01\); effect size: \(\Delta \sigma_{Med}\) = −0.21 s; 99% CI (−0.60 s, −0.21 s)).

**\(Ca^{2+}\) waves transition from random multiple initiation sites to preferred sites as \(Ca^{2+}\) increases in computational models incorporating heterogeneity**

Unlike the data in Fig. 1, we found previously that in a simulated homogeneous CRU network, wave initiation
sites were distributed randomly at both low and high Ca$^{2+}$, even though the ISI decreased and became more periodic at high Ca$^{2+}$ (Nivala et al. 2012b). However, in a real myocyte, the CRU network contains heterogeneities (Soeller et al. 2007; Baddeley et al. 2009), both in the size and composition of individual CRUs and in their spatial distribution relative to each other and various intracellular organelles. To test if a simplified heterogeneity in the CRU network could qualitatively account for the experimental observation that Ca$^{2+}$ wave initiation sites become less random as free Ca$^{2+}$ in the bath solution increases (Fig. 1), we introduced a heterogeneous RyR cluster distribution into our 3-D spatially distributed Ca$^{2+}$ cycling model by increasing the number of RyRs per CRU from 100 to 110 in all of the CRUs located in the left 20% of the total cell length (i.e. the 20 × 20 × 10 leftmost CRUs), and observed wave behaviour at various [Ca$^{2+}$]$_i$. Figures 2A and B show linescans of free cytoplasmic Ca$^{2+}$ concentration at two different [Ca$^{2+}$]$_i$ in the myocyte model (see Supplemental Movies for spatiotemporal dynamics). At a low [Ca$^{2+}$]$_i$, just beyond the transition to full wave behaviour, waves occurred sporadically at random locations. At the higher [Ca$^{2+}$]$_i$, however, waves became more periodic and originated consistently from
the heterogeneity at the left end of the cell, which acted as a pacemaker site entraining whole-cell oscillations. This agrees with the experimental observations in Fig. 1. To examine this process in a more systematic fashion and to facilitate the collection of wave initiation statistics, we performed additional analyses using a quasi-2-D version of our spatially distributed Ca\(^{2+}\) cycling model, since the full 3-D model was computationally too demanding. The 2-D heterogeneous model consisted of 100 \(\times\) 20 CRUs, in which the left 20 \(\times\) 20 CRUs contained 110 RyRs per CRU, and the remaining 80 \(\times\) 20 CRUs contained 100 RyRs per CRU. Although this heterogeneity was admittedly not physiologically realistic, it served the purpose of creating a large region of the cell with different sensitivity to Ca\(^{2+}\). We then studied how the heterogeneity influenced the wave behaviour over a wide range of [Ca\(^{2+}\)]\(_o\). Specifically, we measured the extent to which the heterogeneity entrained the whole-cell oscillations by recording the percentage of waves initiated on the left versus the right half of the cell for roughly 100 waves, with any deviation from a 50/50 left/right distribution indicating an effect due to heterogeneity. At a low [Ca\(^{2+}\)]\(_o\) just beyond the transition into the wave regime, the left/right initiation distribution was roughly 60/40, indicating that the heterogeneous region only modestly influenced the wave site generation process (Fig. 2C). At increasingly higher [Ca\(^{2+}\)]\(_o\), the left/right distribution shifted to an increasingly more left dominant pattern, indicating that the degree of entrainment by the identical heterogeneity depends on the Ca\(^{2+}\) level of the cell. We also examined the effect of changing the size of the heterogeneity at a fixed [Ca\(^{2+}\)]\(_o\) (Fig. 2D) and observed

![Figure 2. Ca\(^{2+}\) wave initiation sites in a 3-D cell model](image)

Figure 2. Ca\(^{2+}\) wave initiation sites in a 3-D cell model

A. [Ca\(^{2+}\)]\(_o\) = 3.1 mM, B, [Ca\(^{2+}\)]\(_o\) = 10 mM. Arrows indicate the wave initiation sites. A heterogeneous region (20 \(\times\) 20 \(\times\) 10 CRUs) was included in the left of the network by increasing the number of RyRs from 100 to 110. C, percentage of waves that initiated from the left half of the 100 \(\times\) 20 CRU network model versus extracellular Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_o\). A heterogeneous region was included in the left 20 \(\times\) 20 CRUs (see inset) by increasing the number of RyRs from 100 to 110. D, percentage of the waves that initiated from the left half of the 100 \(\times\) 20 CRU network model versus the size of the heterogeneous region. The percentages in C and D are only approximate due to limited run time (roughly 100 total waves).
that for the given conditions, there was a critical size of heterogeneity necessary in order for entrainment to occur. For example, for $[\text{Ca}^{2+}]_o = 10 \text{ mM}$, the entrainment rate increased dramatically to >85% when the size of the heterogeneity reached $5 \times 5 \text{ CRUs}$.

**Ca}^{2+}$$ oscillations as an emergent behaviour of coupled CRUs**

To understand how the heterogeneity becomes a progressively more effective pacemaker region entraining Ca}^{2+}$$ waves as the Ca}^{2+}$$ level increases (Fig. 2), we first studied how Ca}^{2+}$$ oscillations emerge in coupled CRU arrays under different conditions. We used the quasi-2-D CRU network model and carried out simulations by varying the sizes of the networks (e.g. $1 \times 1 \text{ CRU}, 2 \times 2 \text{ CRUs, ...}$) to study the spiking properties (e.g. the ISI distribution, its average ($\langle \text{ISI} \rangle$) and the standard deviation (SD) of the whole system.

Figure 3A shows the $\langle \text{ISI} \rangle$ and SD versus the number of coupled CRUs for $[\text{Ca}^{2+}]_o = 3.1 \text{ mM}$. For a single isolated CRU, the $\langle \text{ISI} \rangle$ was about 5 s with a SD of 2.5 s. As the number of coupled CRUs increased, both the $\langle \text{ISI} \rangle$ and SD increased. For example, in a $20 \times 20 \text{ CRU}$ array, the $\langle \text{ISI} \rangle$ was around 20 s with a SD of 13 s. As the number of coupled CRUs increased further, the two quantities saturated at large array sizes. Figure 3B shows the averaged whole-system cytosolic Ca}^{2+}$$ concentrations ($[\text{Ca}^{2+}]_i$) versus time for a single uncoupled CRU and a 2-D array of $100 \times 20 \text{ coupled CRUs}$. Two important features should be noted. The spark interval in a single uncoupled CRU was much shorter than the Ca}^{2+}$$ wave oscillation interval (ISI) in a coupled system. This same phenomenon was demonstrated in recent experiments in cultured human neuroblastoma SH-SY5Y cells by Thurley et al. (2011), who showed that the inter-puff intervals were much shorter than the ISIs at the whole-cell level. Another important feature is that the whole system exhibits a much larger random fluctuation than a single unit. This also shows how single channel noise can cause large macroscopic variations at the cellular scale. This random nature of Ca}^{2+}$$ oscillations at the whole-cell level has been shown and analysed in studies by Skupin et al. (2008) in different cell types and investigated in simulations (Skupin et al. 2010).

However, at high $[\text{Ca}^{2+}]_o$, the behaviours are different. Figure 3C shows the $\langle \text{ISI} \rangle$ and SD for $[\text{Ca}^{2+}]_o = 10 \text{ mM}$. For a single uncoupled CRU, the $\langle \text{ISI} \rangle$ was shorter than at
low $\text{Ca}^{2+}$, averaging about 2.3 s with a SD of 1.1 s. Both the $<\text{ISI}>$ and SD decreased as the number of CRUs increased and tended to saturate as the system size became large. Figure 3D shows the cytosolic $\text{Ca}^{2+}$ concentrations versus time for a single CRU and an array of 100 $\times$ 20 coupled CRUs. Contrary to the low $\text{Ca}^{2+}$ case, CRU coupling reduced randomness and accelerated the frequency of the $\text{Ca}^{2+}$ wave oscillations. In other words, the $\text{Ca}^{2+}$ wave oscillation frequency of the coupled system was faster and more periodic than that of the $\text{Ca}^{2+}$ spark frequency of a single uncoupled element. This type of frequency enhancement has also been shown theoretically in coupled random FitzHugh–Nagumo oscillators (Chiang et al., 2009a) and used to explain the frequency enhancement observed in cultured cardiac monolayers (Chen et al., 2009).

Figure 4 summarizes the $<\text{ISI}>$ and SD versus $[\text{Ca}^{2+}]_o$ for a single CRU, an array of 20 $\times$ 20 CRUs, and an array of 100 $\times$ 20 CRUs, illustrating the transition from frequency suppression to frequency enhancement as $[\text{Ca}^{2+}]_o$ increased. As $[\text{Ca}^{2+}]_o$ increased from 3.1 mM to 10 mM, the $<\text{ISI}>$ of the single CRU decreased from 5 s to about 2.5 s, and the SD from 2.3 s to 1.1 s, almost linearly. In the coupled networks, the $<\text{ISI}>$ decreased exponentially from around 20 s to below 2 s, and so did the SD. The network switched from frequency suppression to frequency enhancement at around $[\text{Ca}^{2+}]_o = 6$ mM, i.e. for $[\text{Ca}^{2+}]_o < 6$ mM, the frequency was lower in the coupled networks than that of a single uncoupled CRU, but for $[\text{Ca}^{2+}]_o > 6$ mM, the frequency was higher in the coupled networks.

Besides altering the $\text{Ca}^{2+}$ level, this same behaviour was reproduced by changing other parameters. Figure 5 shows results obtained by changing the number of RyRs per CRU. For the low $[\text{Ca}^{2+}]_o$, the $<\text{ISI}>$ decreased almost linearly from 8 s to 4.5 s in the single uncoupled CRU as the number of RyRs per CRU increased from 20 to 120, accompanied by a similar linear decay in SD (Fig. 5A). In an array of 20 $\times$ 20 CRUs (Fig. 5B), no waves were observed if the number of RyRs per CRU was smaller than 70, and the $<\text{ISI}>$ and SD decayed very non-linearly with the RyR number per CRU, from 90 s to 13 s, as the RyR number per CRU increased from 75 to 120. For the high $[\text{Ca}^{2+}]_o$, the $<\text{ISI}>$ decreased almost linearly from 3 s to 2 s as the RyR number per CRU increased from 20 to 120 (Fig. 5C). In an array of 20 $\times$ 20 CRUs (Fig. 5D), the $<\text{ISI}>$ was longer and SD larger than those of the single CRU when the RyR number per CRU was less than 50, but the $<\text{ISI}>$ was shorter and SD smaller than those of the single CRU when the RyR number per CRU was greater than 50.

We also compared the ISI histograms for both low and high $[\text{Ca}^{2+}]_o$, using very long simulations. Panels A–C of Fig. 6 show the results for high $[\text{Ca}^{2+}]_o$. In Fig. 6A and B, the ISI distribution of a single CRU (Fig. 6A) is non-Gaussian, resembled by a gamma distribution, whereas the ISI distribution for a 20 $\times$ 20 CRU network (Fig. 6B) was well-described by a Gaussian distribution. The range of ISI in the CRU network was much narrower than that for the single CRU. The return map of ISI for the CRU network (Fig. 6C) showed no structure, indicating that the consecutive firings were independent. Panels D–F of Fig. 6 show the results for the low $[\text{Ca}^{2+}]_o$. The ISI of the single CRU (Fig. 6D) was random and ranged from 0.3 to 14 s. The ISI of the CRU network (Fig. 6E), however, peaked at a value 10 times longer than that of the single CRU and had a fat tail. The return map (Fig. 6F) again showed no structure. The fat-tailed distribution in the low $\text{Ca}^{2+}$ case resulted from runs of firings interspersed with occasional long pauses (see Fig. 3B). Note that at both low and high $\text{Ca}^{2+}$, the variation in firing frequency of the single uncoupled CRU did not differ substantially, whereas their variation in the coupled network did. This indicates that these differences in firing properties emerged as a result of coupling between CRUs and dynamical organization, rather than the intrinsic properties of the single CRU per se.

Random entrainment in heterogeneous CRU networks

Unlike a system of two deterministic coupled oscillators with resettable phases where the fastest oscillator always entrains the slowest one (Guevara & Glass, 1982; Zeng et al., 1991), entrainment in a random system is probabilistic and depends critically on the degree of overlap between the individual ISI distributions. To distinguish the latter case from a deterministic oscillator, classically known as a limit cycle, we adopt the term ‘random oscillator.’ As shown above, groups of CRUs, which do not limit cycles.
themselves, can self-organize into random oscillators, allowing us to recast our heterogeneous CRU network in a simplified manner as a system of two coupled random oscillators by imagining the heterogeneity (e.g. the 20 × 20 CRUs with the higher RyR density) as one random oscillator, and the rest of the CRU network as a second random oscillator. As we show below, the differences between ISI distributions of the two random oscillators at low and high [Ca\(^{2+}\)]o account for the increasing degree of entrainment by the heterogeneity as Ca\(^{2+}\) increases.

Consider a system of two coupled random oscillators, and denote one random oscillator by F (for fast) and the other random oscillator by S (for slow), with ISI probability distributions \(p_F(x)\) and \(p_S(x)\), respectively (Fig. 7A). Assume that when one fires, it entrains the other with no delay. Then the probability of S entraining F is:

\[
p_{FS} = \int_{0}^{\infty} p_F(y) \int_{0}^{y} p_S(x) \, dx \, dy.
\]  

Specifically, eqn (5) can be obtained as follows. The probability that F will fire at time \(y\) after the previous firing is \(p_F(y)\). However, any firing from S earlier than \(y\) will entrain site F. Therefore, the conditional probability that a predicted firing of F at time \(y\) does not occur is \(p_{SF}(y) = p_F(y) \int_{0}^{y} p_S(x) \, dx\). Summing up all possible firing intervals in site F, one obtains eqn (5). Similarly, the probability of F entraining S is:

\[
p_{FS} = \int_{0}^{\infty} p_S(y) \int_{0}^{y} p_F(x) \, dx \, dy.
\]

These two equations form the basis of our theory of random entrainment.

One can show that if F and S are Gaussian-distributed with means \(\mu_F\) and \(\mu_S\), and SDs \(\sigma_F\) and \(\sigma_S\), respectively, then the probability that a random number chosen from distribution F is greater than one chosen from S is also Gaussian-distributed with mean \(\mu_F - \mu_S\) and SD \(\sqrt{\sigma^2_F + \sigma^2_S}\), with \(p_{FS}\) equal to the area under the curve in the left-half plane. Using this formulation, one can show that F entrains S only as \(\mu_F - \mu_S\) surpasses \(\sqrt{\sigma^2_F + \sigma^2_S}\).

Figure 8B shows the percentage of firing from F versus
the shift \(\Delta (=\mu_1-\mu_2)\) for different SDs \(\sigma\), showing that the entrainment increases with \(\Delta\) and decreases with \(\sigma\). The results are intuitive, i.e. for larger \(\Delta\) or smaller \(\sigma\), the two distributions overlap less, and thus the fast one can better entrain the slow one. This simple result may be sufficient to explain the observations in experiments and in our simulations that Ca\(^{2+}\) waves tended to originate from roughly the same location at high Ca\(^{2+}\) but occurred randomly in space and time at low Ca\(^{2+}\). That is, the SD at low Ca\(^{2+}\) is much larger than that at high Ca\(^{2+}\). Even though the difference in firing frequency is also larger, the very large SD at low Ca\(^{2+}\) causes the fast region to fail to entrain the slow region.

In addition to the broadness of the distribution, the fat tail of the ISI distribution at low Ca\(^{2+}\) levels may also contribute to the reduction of entrainment. The long ISIs in the fat tail allow short firings to occur from the slow site and entrain the fast site, reducing the efficacy of the fast site entraining the slow site. To study this effect, we truncated the Gaussian distribution at the right side and added a fat tail (in the form \(C(x-x_0)^{-1}\)). Figure 7C shows the percentage of firing from the fast site versus the joint point \((x_1)\) of the combined distributions, showing that adding the tail reduces the entrainment. We also performed computer simulations using the ISI data for the computer simulation of a CRU network shown in Fig. 6E, showing that cutting the fat tail enhances entrainment (Fig. 7D).

**Discussion**

The transition from Ca\(^{2+}\) sparks to Ca\(^{2+}\) waves and oscillations has been the subject of many experimental (Cheng et al. 1996; Parker et al. 1996; Bootman et al. 1997; Wier et al. 1997; Callamara et al. 1998; Marchant et al. 1999; Marchant & Parker, 2001) and simulation (Falcke, 2003; Izu et al. 2006; Skupin & Falcke, 2009; Skupin et al. 2010; Nivala et al. 2012b) studies. In our previous study (Nivala et al. 2012b), we showed that, in the transition from sparks to waves, spark clusters self-organize due to CICR. This process exhibits a power-law distribution, which is a hallmark of criticality.
a dynamical phenomenon widely studied in statistical physics (Stanley, 1999) and in many natural systems (Bak, 1997). In this study, we used computer simulation to further study the dynamics underlying the transition from sparks to waves and global cell-wide oscillations by comparing the Ca$^{2+}$ release dynamics in a single CRU to that in coupled CRU networks with or without subcellular heterogeneities. Our simulation results and theoretical analyses provide mechanistic insights into a variety of experimental observations relating to the origin of Ca$^{2+}$ waves underlying Ca$^{2+}$ oscillations and pacemaking by Ca$^{2+}$ clocks in biological cells.

**Dynamical organization in spark-to-wave transitions and oscillations**

A Ca$^{2+}$ spark may cause a neighbouring CRU to fire via Ca$^{2+}$ diffusion and CICR, which may then cause its neighbours to fire, and so on. If this chain process is persistent, a Ca$^{2+}$ wave forms and propagates. This is commonly observed in experiments and is considered as the mechanism by which Ca$^{2+}$ sparks initiate Ca$^{2+}$ waves (Cheng et al. 1996; Wier et al. 1997; Keizer & Smith, 1998; Keizer et al. 1998). However, the persistence of the chain of spark-induced sparks may not be sustainable due to the random latency of CRU firing and the recovery of neighbouring CRUs from spontaneous firings. In fact, the vast majority of sparks in a cell do not initiate Ca$^{2+}$ waves (Cheng et al. 1996; Wier et al. 1997). Our previous study (Nivala et al. 2012b) showed that the spark-to-wave transition is similar to a second-order phase transition in statistical physics. At very low Ca$^{2+}$ levels, Ca$^{2+}$ sparks occur randomly and sparsely, and thus, no macroscopic Ca$^{2+}$ events occur. As Ca$^{2+}$ increases, Ca$^{2+}$ sparks form clusters with the cluster size distribution exhibiting a power-law, inducing large fluctuations in the local Ca$^{2+}$ concentration, which eventually trigger macroscopic Ca$^{2+}$ waves. As Ca$^{2+}$ increases further, CRUs fire in a more synchronous manner, and the global Ca$^{2+}$ signal becomes more periodic. This provides a formal theoretical basis for the spark-to-wave transitions and long-range correlations observed in intracellular Ca$^{2+}$ signalling.

In this study, our computer simulations show that the spiking behaviour of groups of CRUs cannot be decomposed into the individual dynamics of the CRUs themselves. When the interaction between CRUs is weak (e.g. at low Ca$^{2+}$ levels or low RyR or IP$_3$ receptor density), the frequency of global firing in a CRU network is much slower than that of a single CRU. This indicates that the organization process for wave initiation dominates over the time dynamics of single CRU firings (sparks), implying that a dynamical time scale for sparks to self-organize into global events has emerged from the CRU network. In other words, the time scale to form a cluster of sufficient size to initiate a Ca$^{2+}$ wave is very long compared to the sparking rate of individual CRUs, so that a CRU is more likely to be activated as a primary spark and only occasionally as a secondary spark triggered by a Ca$^{2+}$ wave. This directly

**Figure 7. Theoretical analyses for random entrainment**

A, illustration of the distribution setup used in B. B, percentage of firing originating from the fast site versus $\Delta$ for different standard deviations (labelled) for Gaussian distribution: $p(x) = \frac{1}{\sigma \sqrt{2\pi}} e^{-\frac{(x - \mu)^2}{2 \sigma^2}}$ (x is in units of seconds), obtained using eqns 5 and 6. $\mu$ = 2 s. C, percentage of firing originating from the fast site versus $x_0$ (as indicated in the inset) for asymmetric distribution. $\Delta$ = 1.5 s. The Gaussian distribution function is: $N(x) = C e^{-\frac{(x - \mu)^2}{2 \sigma^2}}$ and the fat tail is: $h(x) = C_2 (x - x_0)^{-\alpha}$ (x is in units of seconds). D, percentage of firing originating from the fast site versus the shift $\Delta$ from simulations using data from the histogram in Fig. 6E. Specifically, we assume that the two firing sites have the same ISI histogram as in Fig. 6E, one with a shift of $\Delta$. In the case of fat-tail cutting (red curve), the ISIs greater than 25 s were dropped.
agrees with the experimental observations in cultured human neuroblastoma SH-SY5Y cells by Thurley et al. (2011). As shown by our statistics (Fig. 6E), this dynamic time scale exhibits a fat-tailed distribution, a hallmark of self-organization and criticality.

At high $\text{Ca}^{2+}$ levels, on the other hand, the global firing frequency is faster than the individual firing frequency of a single uncoupled CRU. Since at high $\text{Ca}^{2+}$, RyRs are more sensitive, a firing CRU has a high probability of recruiting its neighbours to fire, accelerating the global firing frequency. In this case, the dynamic time scale is limited by RyR recovery time and SR refilling time. In other words, the time scale to form a cluster of sufficient size to initiate a $\text{Ca}^{2+}$ wave becomes shorter than the sparking rate of an isolated uncoupled CRU, such that most CRUs are firing spontaneously as primary sparks. This acceleration of firing in coupled CRU networks at high $[\text{Ca}^{2+}]_o$ is similar to the frequency enhancement shown in coupled random FitzHugh-Nagumo oscillators (Chiang et al. 2011) in which the frequency of the coupled oscillators is faster than the uncoupled ones. Note that altering $[\text{Ca}^{2+}]_o$ or RyR density alters the firing frequency of the single CRU, mostly in a linear manner, but the effect on the global firing frequency is much larger and non-linear, indicating that the interaction (or coupling) between the CRUs plays an important role. The coupling effect can be directly demonstrated in computer simulations by changing the distance between CRUs, i.e. the closer the CRUs, the stronger the coupling. Figure 8A shows an ISI histogram from a $20 \times 20$ CRU network for low $\text{Ca}^{2+}$ ($[\text{Ca}^{2+}]_o = 3.1 \text{ mM}$), in which the CRU spacing was changed from 1 $\mu$m to 0.8 $\mu$m. The $<\text{ISI}>$ became much shorter (from $\sim 20$ s to $\sim 8$ s), and the SD much smaller (from $\sim 13$ s to $\sim 1$ s). The distribution lost its fat tail and resembled a Gaussian distribution. The effect of CRU spacing on $\text{Ca}^{2+}$ wave formation was studied previously in computer simulations by Izu et al. (2006), with similar results.

**Roles of subcellular heterogeneities**

In uniform models such as the CRU network simulated in our previous study (Nivala et al. 2012b), $\text{Ca}^{2+}$ waves organized randomly in space. However, real cells are not uniform and heterogeneities exist, for example, in the number of RyRs (or IP$_3$ receptors) per CRU, distances between neighbouring CRUs, and discontinuities caused by other intracellular organelles and membrane invaginations (Seoeller et al. 2007; Baddeley et al. 2009). In heterogeneous deterministically excitable systems, the fastest firing site (even if it is only slightly faster than the others) always entrains the whole system, unless conduction breaks occur between firing sites (Xie et al. 2001b; Qu & Weiss, 2005). In heterogeneous randomly excitable systems, however, the randomness of the firings allows entrainment to be intermittent. As shown in our theoretical analysis, the rate of entrainment depends not only on the difference in firing rates, but also on the broadness and the shape of the ISI distribution. In our simulations, the ISI distribution is much narrower at high $\text{Ca}^{2+}$ than at low $\text{Ca}^{2+}$, making entrainment much more efficient at high $\text{Ca}^{2+}$, explaining our experimental observations. Furthermore, the fat-tailed ISI morphology that results from a system being near criticality significantly contributes to the failure of entrainment at low $\text{Ca}^{2+}$.

In heterogeneous excitable systems, the spatial scale of heterogeneity plays an important role for the wave behaviours (Xie et al. 2001a). As shown in Fig. 2D, the entrainment depends on the size of the heterogeneity. In fact, if we increase the number of RyRs from 100 to 110 in 20% of CRUs whose spatial location is chosen randomly from a uniform distribution, then entrainment is lost, and wave dynamics are similar to a homogeneous system. We also used a Gaussian distribution of RyR cluster size (with an average 100 RyRs and standard deviation 10) in our simulation and failed to observe the formation of stable pacemaker sites at high $\text{Ca}^{2+}$. Instead, we still observed pacemaker sites occurring randomly in space as in the homogeneous system. Therefore, the spatial

**Figure 8. Effects of decreased CRU spacing on ISI and entrainment at low $\text{Ca}^{2+}$ ($[\text{Ca}^{2+}]_o = 3.1 \text{ mM}$)**

A, ISI histogram from a $20 \times 20$ CRU network with CRU spacing 0.8 $\mu$m. The red curve is a Gaussian distribution function. $f(x) = 200e^{-\frac{(x - 9)^2}{2}}$ (x is in units of seconds). B, $\text{Ca}^{2+}$ wave initiation sites in a $100 \times 20$ CRU network cell model. Arrows indicate the wave initiation sites. A heterogeneous region ($25 \times 25$ CRUs) with CRU spacing decreased from 1.0 $\mu$m to 0.8 $\mu$m was included in the left of the network.
scale or correlation of RyR cluster size heterogeneity is important for the formation of stable pacemaker sites. Since, to our knowledge, the spatial correlation of RyR cluster size in cardiac myocytes has yet to be characterized experimentally, we chose to use a very simple yet extreme form of heterogeneity to study the effects of heterogeneities in CRU networks in the present study. Even though our heterogeneous CRU network model is not physiologically realistic, this extreme case is useful because it demonstrates that even with such an extreme heterogeneity, the effects of the heterogeneity are minimal at low Ca$^{2+}$ and are only unmasked at high Ca$^{2+}$. We believe that the general mechanisms of random entrainment will still hold in more realistic heterogeneous systems. In addition, real cells most likely contain multiple heterogeneities, and our theory of random entrainment is easily generalized to more than two coupled random oscillators. In such cases, the degree to which each pacemaker region entrains cellular oscillations depends on the relative overlap of multiple ISI distributions, and multiple pacemaker sites can co-exist. It should be noted that the scenario in which entrainment occurs at high Ca$^{2+}$ but fails at low Ca$^{2+}$ is only relative. If the system is highly heterogeneous such that the heterogeneous region has a much narrower distribution and faster frequency than other regions, entrainment can still occur at low Ca$^{2+}$. This is demonstrated in a simulation shown in Fig. 8, in which we made the CRU spacing shorter (from CRU spacing $1 \mu\text{m}$ to $0.8 \mu\text{m}$) at low Ca$^{2+}$ and found that almost all firings originated from the heterogeneous region. In this case, entrainment occurs because the ISI is shorter and much less variable than for control CRU spacing.

It is also important to note that individual CRUs in a heterogeneous region do not oscillate periodically as limit cycles when uncoupled. Periodic pacemaking is still a self-organized phenomenon of a group of randomly firing CRUs. As shown in our simulations, when the coupled CRU network exhibits a strong periodicity, the frequency is faster and much less variable than a single CRU. Thus, the periodicity of the pacemaker site depends strongly and directly on the coupling between CRUs.

**Implications for Ca$^{2+}$ cycling dynamics in the heart**

Intracellular Ca$^{2+}$ cycling in cardiac systems is not only required for excitation–contraction coupling but also plays important roles in generating both the normal heart rhythm under physiological conditions (Lakatta et al. 2010) and life-threatening arrhythmias under pathophysiological conditions of Ca$^{2+}$ overload (ter Keurs & Boyden, 2007). Results from this and our previous study (Nivala et al. 2012b) may provide new mechanistic insights into these dynamics.

For example, heart rate variability during sinus rhythm has been shown to exhibit power-law (fractal) behaviours (Kobayashi & Musha, 1982; Ivanov et al. 1996). This feature is often attributed to autonomic tone, but cardiac preparations which lack autonomic innervation, such as cultured neonatal cardiac monolayers (Kucera et al. 2000; Ponard et al. 2007), also exhibited a power-law distribution of beating intervals. Although simulations suggested that a Poisson distribution of ion channel protein turnover fluctuations could give rise to power-law behaviour of heart rate variability in this setting, our findings may provide an alternative mechanism related to Ca$^{2+}$ cycling, which is now recognized, together with voltage-dependent oscillations, to play a fundamental role in sino-atrial nodal pacemaking (Vinogradova & Lakatta, 2009; Vinogradova et al. 2010). Prior to the action potential upstroke, the occurrence of local Ca$^{2+}$ releases increases inward Na$^{+}$–Ca$^{2+}$ exchanger current to enhance diastolic depolarization. The local Ca$^{2+}$ releases occur more or less randomly in space and time, and their periods, which vary from beat to beat, correlate linearly with the cycle lengths of sino-atrial nodal cell oscillations. The local Ca$^{2+}$ releases are not random single sparks but rather self-organized spark clusters, as also demonstrated in a recent modelling study (Maltev et al. 2011). It is intriguing to speculate that during the transition from Ca$^{2+}$ sparks to whole-cell oscillations, the power-law spark clustering and fat-tailed ISI distribution occur via criticality, potentially contributing to the power-law behaviour in heart rate variability. This is a promising area for future sino-atrial nodal cell and cell network modelling.

Under pathophysiological conditions in atrial and ventricular myocytes and in Purkinje cells, Ca$^{2+}$ waves activate Ca$^{2+}$-sensitive inward currents such as Na$^{+}$–Ca$^{2+}$ exchanger to cause delayed after-depolarizations (DADs), which are arrhythmogenic (ter Keurs & Boyden, 2007). In multicellular tissue, however, a Ca$^{2+}$ wave from a single myocyte cannot generate enough inward current to cause a DAD since its resting voltage is clamped by the neighbouring myocytes without Ca$^{2+}$ waves (Fuijwara et al. 2008). To overcome this source–sink mismatch, Ca$^{2+}$ waves have to occur synchronously in tens to thousands for a DAD to occur (Xie et al. 2010). Our results may help to explain how this happens, since as Ca$^{2+}$ increases, the <ISI> and SD of Ca$^{2+}$ waves both decrease markedly (Fig. 2). In coupled myocytes with similar Ca$^{2+}$ cycling properties, this would naturally lead to a synchronization of the timing of Ca$^{2+}$ waves in neighbouring myocytes as the Ca$^{2+}$ load increased, as observed in multicellular tissue when Ca$^{2+}$ loading has been varied by rapid pacing (Fuijwara et al. 2008; Wasserstrom et al. 2010). In failing hearts or genetic models of catecholaminergic polymorphic ventricular tachycardia, RyRs are more sensitive to Ca$^{2+}$ and thus leakier. Even though SR Ca$^{2+}$ load is lower, the increase in RyR sensitivity may enhance CRU coupling, which facilitates Ca$^{2+}$ wave formation and DADs. Moreover, in failing myocytes, the CRU spacing
becomes shorter (Chen-Izu et al. 2007) and facilitates wave formation. Heterogeneity may also be enhanced in failing myocytes due to T-tubule disruption and disorganization to promote Ca\(^{2+}\) waves.

An important question is what determines the timing of the occurrence of Ca\(^{2+}\) waves in cardiac myocytes. Sobie et al. (2006) and Ramay et al. (2011) showed that the time constant of recovery of Ca\(^{2+}\) spark amplitude is \(\sim 100\) ms, and the intervals between consecutive sparks are on the order of a couple of hundred milliseconds. The recovery time constant is determined by SR refilling and RyR sensitivity. Brochet et al. (2005) have shown that the local SR refilling time is less than 100 ms. Therefore, if the occurrence of Ca\(^{2+}\) waves was determined by the refractory period and SR refilling time, Ca\(^{2+}\) waves should occur at intervals on the order of a couple of hundred milliseconds. However, Ca\(^{2+}\) waves occur at intervals of seconds or even longer, which are much longer than the SR refilling time and the recovery time of the CRU, indicating that the occurrence of Ca\(^{2+}\) waves is not solely determined by CRU refractoriness and SR refilling. In a recent experimental study, Belevych et al. (2012) demonstrated that Ca\(^{2+}\) waves occur in a certain time period, which they call the ‘idle period,’ after the RyRs are recovered from inactivation and the SR is refilled. Our study shows that Ca\(^{2+}\) waves are emergent properties of the CRU network, which depends not only on the properties of the individual CRUs, but also strongly on the coupling between CRUs. The idle period identified by Belevych et al. is thus the time for sparks to self-organize into waves, i.e. the idle period is the self-organization period for a critical size of spark clusters to form and initiate a wave.

**Limitations**

The Ca\(^{2+}\) cycling model in this study was developed based on the ventricular myocyte and may be too specific to extrapolate the details of Ca\(^{2+}\) cycling dynamics in other types of cells in which the CRUs consist of IP\(_3\) receptors (Falcke, 2003; Shuai & Jung, 2003). Conversely, the detailed subcellular structure of ventricular myocytes and regulation of CRUs are much more complex than the simple heterogeneities modelled in this study (Soeller & Cannell, 1999; Soeller et al., 2007; Baddeley et al., 2009; Haake et al., 2012). The CRUs are identical in our models, but in real cells, even in ventricular myocytes, they are not. For example, CRUs close to the cell surface exhibit different sarcolemma ion channels than those deep inside the cell. These may cause additional interesting dynamics. In addition, different RyR models (Jafri et al. 1998; Stern et al. 1999; Sobie et al. 2002; Restrepo et al. 2008; Chen et al. 2009d) may give rise to different whole-cell Ca\(^{2+}\) cycling dynamics, which need to be further investigated in modelling studies. The computational simulations were done using a model of a myocyte with an intact sarcolemma in which Ca\(^{2+}\) content in the cell was changed by altering \([\text{Ca}^{2+}]_o\). Experimentally, however, it is difficult to control the Ca\(^{2+}\) level in an intact cell by changing \([\text{Ca}^{2+}]_o\). Therefore, the experiments were performed on permeabilized cells, allowing intracellular Ca\(^{2+}\) to be directly controlled, after verifying that similar results were obtained in the model when the sarcolemma was removed to simulate a permeabilized cell. We feel justified in this approach since it was not the purpose of the present study to obtain strict quantitative agreement between the experiments and the modelling but rather to develop a general dynamical theory for pacemaker generation in cardiac myocytes.

Despite these limitations, our present study provides novel mechanistic insights into how the microscopic random events of intracellular Ca\(^{2+}\) cycling (Ca\(^{2+}\) sparks) integrate into global events like Ca\(^{2+}\) waves and oscillations in cardiac myocytes and in other cell types, as well as general insights into the collective dynamics of coupled random excitable elements.

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Additional information

Competing interests
None.

Author contributions
All authors contributed to the research design, data analysis,
manuscript drafting and revision; M.N. and M.N. carried out the
computer programming and simulations; C.K. did the myocyte
experiments and statistical analysis; M.N. and Z.Q. developed
the theory; Z.Q. conceived the overall research. All authors have
read and approved the final version of the manuscript.

Funding
This work was supported by National Institute of Health
grant P01 HL078931; American Heart Association, Western
States Affiliate, Postdoctoral Fellowship Award 10POST3210024
(M.N.), Predoctoral Fellowship Award 10PRE3030052 (C.K.);
the Laubisch and Kawata Endowments; and the UCLA Cardio-
vascular Development Fund (M.N.).

Acknowledgements
We thank Alan Garfinkel, Hrayr Karagueuzian, Riccardo Olesce,
and the members of the UCLA arrhythmia group for helpful
comments, Rahul D. Patel for cell preparation, and Guillaume
Calmettes for help in statistical analysis.

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The role of spontaneous calcium release on complex EAD morphology
EADs are common arrhythmia triggers that manifest as membrane voltage oscillations in the plateau and repolarization phases of the cardiac myocyte AP. Central to the overall mechanism of EAD emergence is a bidirectional coupling of two mechanistic systems. In the voltage-mediated mechanism, deterministic feedback interactions between the inward $I_{L,Ca}$ and outward K currents, governed by the dynamical Hopf and homoclinic bifurcations, play a primary role in voltage-mediated EAD generation. In the Ca-mediated mechanism, sufficient Ca release from the SR reverses repolarization and promotes EADs by activating primarily $I_{NCX}$. In this context, the property of Ca-voltage coupling raises intriguing possibilities as to the extent to which these two mechanistic systems interact and possibly enhance arrhythmogenicity. As demonstrated at length in the previous chapters, diffusive Ca-mediated interactions between stochastic processes within the CRU network bestow the Ca-mediated mechanism a rich spectrum of spontaneous intracellular Ca release behaviors that give rise to the Ca signaling hierarchy. The role of these Ca release behaviors in promoting arrhythmias is well known. However, very little is understood about how these behaviors directly affect EADs commonly arising from deterministic processes. Therefore, in the following, we will explore how the interaction between the intrinsic dynamics of systolic SR Ca release and voltage-mediated dynamics modulates EAD behavior.
Calcium-Voltage Coupling in the Genesis of Early and Delayed Afterdepolarizations in Cardiac Myocytes

Zhen Song, Christopher Y. Ko, Michael Nivala, James N. Weiss, and Zhilin Qu

ABSTRACT Early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs) are voltage oscillations known to cause cardiac arrhythmias. EADs are mainly driven by voltage oscillations in the repolarizing phase of the action potential (AP), while DADs are driven by spontaneous calcium (Ca) release during diastole. Because voltage and Ca are bidirectionally coupled, they modulate each other’s behaviors, and new AP and Ca cycling dynamics can emerge from this coupling. In this study, we performed computer simulations using an AP model with detailed spatiotemporal Ca cycling incorporating stochastic openings of Ca channels and ryanodine receptors to investigate the effects of Ca-voltage coupling on EAD and DAD dynamics. Simulations were complemented by experiments in mouse ventricular myocytes. We show that: 1) alteration of the Ca transient due to increased ryanodine receptor leakiness and/or sarco/endoplasmic reticulum Ca ATPase activity can either promote or suppress EADs due to the complex effects of Ca on ionic current properties; 2) spontaneous Ca waves also exhibit complex effects on EADs, but cannot induce EADs of significant amplitude without the participation of Ca waves; 3) lengthening AP duration and the occurrence of EADs promote DADs by increasing intracellular Ca loading, and two mechanisms of DADs are identified, i.e., Ca-wave-dependent and Ca-wave-independent; and 4) Ca-voltage coupling promotes complex EAD patterns such as EAD alternans that are not observed for solely voltage-driven EADs. In conclusion, Ca-voltage coupling combined with the nonlinear dynamical behaviors of voltage and Ca cycling play a key role in generating complex EAD and DAD dynamics observed experimentally in cardiac myocytes, whose mechanisms are complex but analyzable.

INTRODUCTION

Early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs) are known to cause arrhythmias in a variety of cardiac diseases, including long QT syndromes (1–3), catecholaminergic polymorphic ventricular tachycardia (CPVT) (4,5), and heart failure (6,7). EADs are voltage oscillations that occur during the repolarizing phases of the action potential (AP). By prolonging the AP duration (APD) regionally, EADs can increase dispersion of refractoriness, forming a tissue substrate vulnerable to reentry. Furthermore, if EADs reach the threshold to propagate out this region as premature ventricular contractions, they can serve as triggers to induce reentry. DADs, on the other hand, are voltage oscillations during diastole. By depolarizing the resting membrane potential regionally, DADs can cause conduction block (8), and, if they reach the threshold for sodium (Na) channel activation, can generate triggered activity (TA) manifesting as premature ventricular contractions that induce reentry.

EADs occur in the setting of reduced repolarization reserve, such as long QT syndromes, and have classically been attributed to voltage-driven oscillations in the repolarizing phase of the AP, although spontaneous sarcoplasmic reticulum (SR) calcium (Ca) release has also been proposed as an important mechanism. DADs, on the other hand, are promoted by Ca cycling disorders, such as CPVT and digitals toxicity, which promote Ca-driven oscillations. Heart failure represents a combination of reduced repolarization reserve and abnormal Ca cycling resulting from electrical and excitation-contraction coupling remodeling processes driven by both genetic transcriptional and posttranslational signaling components. In all of these settings, it is common for EADs and DADs to coexist and influence each other. This is because membrane voltage is strongly affected by Ca-sensitive ionic currents, and, conversely, cellular Ca loading is strongly influenced by voltage-dependent ionic currents, referred to as bidirectional Ca-voltage coupling. Ca-voltage coupling can promote complex AP dynamics in the heart (9). Analyzing the interactions between EADs and DADs (and voltage and Ca-cycling coupling dynamics in general), however, has been challenging, because the Ca cycling dynamics (e.g., Ca waves and oscillations) result from a spatially distributed heterogeneous network of Ca release units (CRUs) in the cell. The Ca waves emanating from various subcellular regions integrate to depolarize diastolic membrane voltage by activating Ca-sensitive inward currents including the Na-Ca exchange (NCX) current (I_{NCX}) and Ca-activated nonspecific cation channels (I_{Ca,Ca}), generating DADs. Similarly, spontaneous SR Ca
release during the AP plateau can potentially trigger EADs (10–13), in concert with voltage-dependent mechanisms generating voltage oscillations. Therefore, to analyze EAD and DAD dynamics realistically requires a detailed cardiac AP model incorporating spatiotemporal Ca cycling that can simulate Ca waves, such as the ones developed recently (14–16), rather than single pool models of Ca cycling.

In this study, we used our AP model incorporating detailed spatiotemporal Ca cycling in a network of diffusively-coupled CRUs to investigate systematically how bidirectional voltage-Ca coupling affects EAD and DAD dynamics. We show the following: 1) Alteration of the Ca transient can either promote or suppress EADs due to its complex effects on Ca-sensitive ionic currents. 2) Spontaneous Ca oscillations have complex effects on the occurrence and behavior of EADs, but cannot generate EADs of significant amplitude without the participation of \( I_{\text{Ca,L}} \); the timing of a rise in intracellular Ca relative to membrane depolarization is not sufficient criteria to indicate that an EAD is caused by spontaneous SR Ca release. 3) Two different DAD mechanisms are identified (Ca-wave-independent and Ca-wave-dependent), and lengthening of APD promotes DADs by increasing intracellular Ca loading. The presence of EADs further enhances Ca loading to promote DADs due to more L-type Ca channel (LCC) openings during EADs. 4) Complex EAD patterns occur due to Ca-voltage coupling and are promoted under Ca overload conditions in both computer simulation and experiments of mouse ventricular myocytes. The mechanisms underlying these different EAD and DAD dynamics are analyzed and discussed.

**MATERIALS AND METHODS**

**Computer model**

The myocyte model contains a three-dimensional network of 19,305 (65 \( \times \) 27 \( \times \) 11) CRUs, modified from Restrepo et al. (14) Each CRU contains five subvolumes: network SR, junctional SR, diadic space, subsarcolemmal space, and cytosolic space. Each CRU has a cluster of 100 ryanodine receptor (RyR) channel clusters associated with a cluster of 12 LCCs, both simulated using random Markov transitions. The governing equation for voltage is

\[
\frac{dv}{dt} = -\left( I_{\text{NaK}} + \sum_{k=1}^{N} I_{\text{LCC}(k)} + I_{\text{Ks}} + N I_{\text{NaK}}(k) \right)
+ I_{\text{NaK}} + I_{\text{LCC}} + I_{\text{NCX}} \right) / C_{\text{m}},
\]

where \( I_{\text{LCC}} \) is the current of a single LCC, \( I_{\text{NaK}} \) is the NCX current of a single CRU, \( N \) is the total number of CRUs in the cell, and \( n \) is the total number of LCCs in a CRU. Because the \( I_{\text{NaK}} \) window current is extremely important in the genesis of EADs (17–21), we reformulated the Markov LCC model to allow the window current to be directly manipulated. This was necessary because the Markov LCC formulation in the AP model by Restrepo et al. (14) has many linked states, and it is not straightforward to alter the window current without affecting other properties of the current. Therefore, we adopted a Hodgkin-Huxley type of formulation modified from the model of Luo and Rudy (22), in which the window current can be directly altered by shifting the steady-state activation and inactivation curves. In addition, the Hodgkin-Huxley-type model can be directly modified based on whole-cell measurements of the channel kinetics. A full description of the computer model and the numerical methods used are presented in the Supporting Materials and Methods.

**Myocyte experiments**

Patch-clamp recordings and optical imaging of Ca in isolated mouse ventricular myocytes were carried out. Details of the experimental methods are described in the Supporting Materials and Methods. All procedures comply with UCLA Animal Research Committee policies.

**RESULTS**

**EAD and DAD generation in the computer model**

A straightforward method of inducing EADs in the model was to shift the voltage activation curve of \( I_{\text{NaK}} \) to more negative voltages to increase the \( I_{\text{Ca,L}} \) window current (17), mimicking the effects of Ca channel mutations that cause long QT syndrome, such as Timothy syndrome (23) and other disease conditions such as hypertrophy (18). This shift had very small effects on \( I_{\text{Ca,L}} \) and the Ca transient under AP clamp conditions (Fig. S4 in the Supporting Material). Under free-running conditions in which the AP was not clamped, however, this small shift in the activation curve lengthened APD and resulted in EADs, which then had much larger effects on \( I_{\text{Ca,L}} \) and the Ca transient. Conversely, a straightforward method of inducing DADs in the model was to raise \([\text{Na}]_o\) such as might occur in chronic heart failure (24); this increased intracellular Ca loading via NCX sufficiently to cause spontaneous SR Ca release during diastole. Elevating \([\text{Na}]_o\) increased the Ca transient that augmented Ca-dependent currents, such as \( I_{\text{NCX}} \) and \( I_{\text{NaK}} \), but also increased outward \( I_{\text{NaK}} \) (Fig. S4).

To study the interactions between EADs and DADs, we varied both parameters simultaneously. Fig. 1 summarizes the spectrum of AP behaviors observed for different hyperpolarizing left-shifts in the \( I_{\text{Ca,L}} \) activation curve (0–9 mV) coupled with different levels of \( [\text{Na}]_o \) (5–15 mM) during pacing at \( \text{PCL} = 2, 1, \) and 0.5 s, respectively. Six typical AP behaviors were observed at all PCLs, illustrated in the sample traces shown in Fig. 1 D: 1) normal AP, 2) EADs only, 3) repolarization failure, 4) DADs only, 5) a mixture of EADs and DADs, and 6) repolarization failure with voltage oscillations. More complex EAD and DAD behaviors, including TA, also occurred under specific conditions, which are discussed in detail later. In general, Fig. 1 A–C, shows that shifting the \( I_{\text{Ca,L}} \) activation curve to more negative voltages promoted EADs and repolarization failure without inducing DADs. On the other hand, increasing \([\text{Na}]_o\) caused DADs by elevating \([\text{Ca}]_i\) and enhancing spontaneous SR Ca waves, but tended to suppress EADs and repolarization failure by increasing outward \( I_{\text{NaK}} \) and reducing inward (forward mode) NCX current. When both
I_{Ca,L} activation was shifted and [Na] was elevated, EADs and DADs commonly occurred together.

The size of the regions exhibiting DADs or EADs varied with PCL. A faster heart rate (PCL = 0.5 s) expanded the DAD region, induced TA, and shrunk the EAD region, whereas a slower heart rate (PCL = 2 s) had the converse effects. The results are consistent with experimental observations that EADs are often promoted by bradycardia, which reduces repolarization reserve, whereas DADs and TAs are often promoted by tachycardia, which exacerbates intracellular Ca loading. The combined EAD + DAD region was largest at the intermediate heart rate (PCL = 1 s). PCL had only minor effects on the normal AP and AP repolarization failure regions.

Modulation of EAD dynamics by Ca cycling

As shown in Fig. 1, A–C, when I_{Ca,L} activation was left-shifted to induce EADs, elevating [Na] promoted DADs but tended to suppress EADs, so that EADs were either replaced by DADs, or coexisted with DADs as [Na] increased. The suppression of EADs occurred because elevating [Na] enhanced outward I_{NaK} and inhibited inward I_{NCX}.

Because altered sarcoplasmic reticulum Ca ATPase (SERCA) activity and RyR leakiness are important features that impact Ca cycling in CPVT and heart failure, we also investigated the effects of these factors on EADs. At PCL = 2 s, doubling SERCA activity strongly promoted DADs but had only small and mixed effects on EADs, as can be seen by comparing the DAD and EAD regions in Fig. 2, A and B. Increasing RyR leakiness also strongly promoted DADs with only a small effect on suppressing EADs (compare Fig. 2, A and C). Combining both alterations enhanced these effects (Fig. 2 D). In this case, the amplitude of DADs also greatly increased, which caused DAD-mediated TA in both the pure DAD and mixed EAD+DAD regions. Because the TA occurred at a shorter cycle length than the PCL (Fig. 3 D), this also contributed to the suppression of EADs.

To illustrate the mechanisms by which SERCA activity and RyR leakiness affected EADs, representative AP and Ca-dependent ionic current traces are presented in Fig. 3. In Fig. 3 A, with [Na] = 8 mM, increasing SERCA activity promoted EADs. The peak of the Ca transient increased due to the greater SR Ca content, but the decay was accelerated due to more rapid SR Ca reuptake. These changes in the Ca transient caused: 1) a reduced peak I_{Ca,L} due to stronger Ca-dependent inactivation, but almost no difference during the plateau phase until the EAD occurred; 2) a small decrease in I_{Ks} in the late plateau phase due to the lower [Ca]; and 3) increased I_{SCX} in the early phase but reduced I_{SCX} in the later phase of the plateau. The voltages were almost identical in the two cases until just before the EAD occurred, but the reduction in I_{Ks} predominated over the reduction in I_{SCX} to promote the EAD. The converse outcome is illustrated in Fig. 3 B when [Na] was raised to
14 mM. In this case, increasing SERCA did not increase the peak of the Ca transient, but still sped up its decay. This change had almost no effect on $I_{Ca,L}$ but reduced both $I_{Ks}$ and $I_{NCX}$. In this case, reduction in $I_{NCX}$ predominated over the reduction of $I_{Ks}$, and the EAD was suppressed.

Fig. 3 illustrates an example in which increasing RyR leakiness suppressed EADs. In this case, the Ca transient was smaller, which had a small effect on peak $I_{Ca,L}$ but reduced $I_{Ks}$ and $I_{NCX}$. Similar to Fig. 3B, the reduction of $I_{NCX}$ predominated, suppressing the EAD. Finally, Fig. 3D shows an example of the combined effects of increasing RyR leakiness and SERCA activity together. In this case, EADs were suppressed and sustained DAD-mediated TA occurred at cycle length < 0.5 s, shorter than the PCL of 2 s.

The results shown above demonstrate that the effects of Ca-voltage coupling are complex and nonintuitive, affecting multiple ionic currents in subtle ways to suppress or potentiate EADs and DADs. For example, in our model, increasing RyR leakiness suppressed EADs due to a smaller Ca transient. This finding generally agrees with previous experimental observations (25,26) that a larger Ca transient under Ca overload conditions tended to promote EADs via increased $I_{NCX}$. On the other hand, a recent study by Terentyev et al. (27) demonstrated that increased RyR leakiness caused a smaller Ca transient but nevertheless promoted EADs in long QT rabbit ventricular myocytes, illustrating the complexity of the interactions.

**Spontaneous Ca oscillations and EADs**

Spontaneous Ca oscillations associated with EADs have been observed in many experimental studies (10,11,13,28,29), and have been interpreted to be the cause of EADs when an intracellular Ca rise precedes the EAD upstroke (28). Supporting this conjecture, other experiments have shown that Ca oscillations associated with EADs can persist after voltage is clamped (13). However, the validity of these criteria for concluding that EADs can be caused by spontaneous Ca release events has not been quantitatively validated. In our computer simulations, we also observed these phenomena, as illustrated in Fig. 4A. Following a long AP with multiple EADs, a subsequent DAD triggered a second AP (marked by an asterisk) with a single EAD (marked by an arrow). Differing from other cases shown earlier (Figs. 1 and 3), the Ca transient during the EAD was much larger and started to rise (~10 ms) before voltage. In particular, the Ca concentration in dyadic space (DS) ([Ca]$_{DS}$) rose much earlier than the EAD upstroke (arrow in Fig. 4B). The line scan of [Ca]$_i$ was also dysynchronous, consistent with multiple spontaneous SR Ca release sites rather than a voltage-driven SR Ca release. These features agree with the above-mentioned experimental observations of spontaneous Ca oscillations and EADs.
Ca release-induced EADs. To investigate the validity of this interpretation, we carried out additional simulations in which we either clamped voltage (Fig. 4B) or clamped intracellular Ca (Fig. 4C) at the EAD onset.

In the voltage-clamp case, voltage was clamped at the onset of the EAD period (marked by the vertical dashed line in Fig. 4B) with a linear decay at different rates. When the voltage decay had a steeper slope (red trace), $[\text{Ca}]_i$ still rose during the clamped period as in the free-running case, although not as rapidly. This indicates that the initial $[\text{Ca}]_i$ rise was not triggered by the EAD, but was due to spontaneous (i.e., non-voltage-driven) Ca release. After the clamp was terminated, voltage repolarized to the resting potential, after which another spontaneous Ca release occurred several hundred milliseconds later, triggering a DAD-induced AP. In contrast, when the voltage decay had a shallower slope (blue trace), $[\text{Ca}]_i$ rose faster than in the steeper case, and after the voltage-clamp was released, both voltage and $[\text{Ca}]_i$ rose sharply due to reactivation of the LCCs (Fig. 4D). Moreover, instead of repolarizing back to the resting potential, the voltage remained at the plateau level due to continuous opening of LCCs. Multiple EADs occurred with a frequency very different from that of the spontaneous Ca release. This is because after the first large release, most CRUs were refractory and could not respond to the first several EADs. Additional simulations at intermediate voltage decay slopes (not shown) defined a critical voltage at the end of the clamped period, below which no reactivation of LCCs occurred, and the cell repolarized to the resting potential. Above this value, reactivation of LCCs occurred, resulting in EADs. These findings indicate that the upstroke of the EAD requires the reactivation of LCCs and cannot be supported solely by $I_{\text{NCX}}$ or other nonregenerative Ca-sensitive currents.

The consequences of clamping intracellular Ca, instead of voltage, during the EAD period are shown in Fig. 4C. Because Ca concentrations varied at different locations in the cell, we clamped the Ca concentration in the DS at their existing values in all 19,305 CRUs, starting at the time when the mean Ca in the DS started to rise (marked by the vertical dashed line in Fig. 4C). We then let Ca in the DS decay linearly at either a fast (blue traces) or slow (red traces) rate. In both cases, despite Ca in the DS being clamped, the voltage remained in the plateau range and oscillated with decrementing amplitude, eventually culminating in repolarization failure. It is interesting to note that a faster Ca decay (blue trace) resulted in smaller EADs and a more stable plateau voltage. This is because when the clamp ended at a lower intracellular Ca, $I_{\text{NCX}}$ was smaller but $I_{\text{Ca,L}}$ became larger (see Fig. 4D) due to less Ca-depended inactivation. This increased window $I_{\text{Ca,L}}$, resulting in EADs and repolarization failure after intracellular Ca was clamped. The converse effect occurred in the free-running case, in which the progressive rise in Ca in the DS caused more $I_{\text{Ca,L}}$ inactivation, which suppressed the window $I_{\text{Ca,L}}$. This resulted in a lower take-off voltage for the EAD, which allowed a stronger voltage-dependent activation of LCCs to give rise to a larger depolarization. From this observation, we conclude although the amplitude of the EAD in the free-running case was potentiated by the increase in Ca preceding the
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[Image 1]

EAD upstroke, the EAD was driven primarily by the LCC reactivation-mediated voltage oscillation, and not underlying Ca oscillations.

These voltage- and Ca-clamp simulations demonstrate the following important mechanistic points. 1) A spontaneous Ca release event preceding the upstroke of an EAD does not necessarily imply that the spontaneous Ca release event is the cause of the EAD; rather, it participates, together with LCC reactivation, in the initiation of the EAD upstroke. 2) The role of spontaneous Ca release is complex—it can suppress EAD amplitude by facilitating Ca-dependent inactivation of LCCs, or enhance EAD amplitude for the same reason depending on the voltage range and balance of other contributing factors.

Modulation of DAD dynamics by voltage

To study the effects of voltage on DADs, we first studied conditions producing a small DAD shown in Fig. 5 A. In an experimental study in canine hearts by Burashnikov and Antzelevitch (30), blocking $I_{Cr}$ prolonged APD, which increased Ca loading sufficiently to induce DADs. Here, we also reduced $I_{Cr}$ to study how APD affects DADs. As $I_{Cr}$ was gradually reduced, APD was prolonged and EADs were induced (Fig. 5 B). The additional Ca loading by the EAD caused the DAD to occur earlier with a larger amplitude (Fig. 5 B, C–F, plots DAD amplitude versus APD (C), DAD coupling interval versus APD (D), DAD amplitude versus SR load (E), and DAD amplitude versus the diastolic [Ca]$_i$ preceding the DAD (F), respectively. The DAD amplitude increased almost linearly with APD in the absence (circles) or the presence (triangles) of EADs. The DAD coupling interval deceased as APD increased. In the absence of EADs, the SR load increased with APD, which in turn increased DAD amplitude.

Note, however, that diastolic [Ca]$_i$ preceding the DAD did not increase significantly. In the presence of EADs, the SR load was near maximal and did not increase further as multiple EADs further prolonged APD. But despite the constant SR load, DAD amplitude continued to increase as APD was prolonged by EADs. The increase in DAD amplitude now tracked the progressive rise in diastolic [Ca]$_i$ preceding the DAD. As shown in the line scans (insets), in the case without EADs, Ca elevation was caused by random CRU firings, while in the case with EADs, Ca elevation was caused by more coordinated CRU firings (due to CRU recruitment). These results indicate: 1) regardless of whether EADs are present, lengthening APD increases Ca loading that promotes DADs and shortens the coupling interval; and 2) two mechanisms of DADs exist: the first depends only on the SR load, and the second depends on both the SR load and the diastolic [Ca]$_i$ level. Note that although APs with or without EADs were used to distinguish between the first and second behaviors in Fig. 5, EADs are not specifically required for the second DAD mechanism to occur. When EADs occur, however, the APD is much longer, enhancing Ca loading. Moreover, more LCCs open during EADs, which further enhances Ca load to potentiate the second mechanism.

Complex EAD dynamics in experiments

Some examples of complex EAD-DAD dynamics observed experimentally are illustrated in Fig. 6, obtained from

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mouse ventricular myocytes loaded with Fluo-4-AM to record 
$[\text{Ca}]_i$ fluorescence. Myocytes were exposed to
 elevated $[\text{Ca}]_o$ (2.7 mM) to induce Ca overload. Fig. 6 A
 shows simultaneous membrane voltage, $[\text{Ca}]_i$ fluorescence,
 and a line scan of $[\text{Ca}]_i$ during an electrically stimu-
 lated AP in a patch-clamped myocyte (current clamp
 mode). Early in the plateau phase of the AP, both $[\text{Ca}]_i$ 
 and voltage fluctuated with small amplitudes. Later, both
 $[\text{Ca}]_i$ and voltage began to oscillate with increasing ampli-
 tude until full repolarization occurred. The line scan of $[\text{Ca}]_i$
 shows that Ca release exhibited random spatiotem-
 poral dynamics in the early plateau phase, which then tran-
 sitioned into synchronized oscillations in the late plateau
 phase.

Similar complex behaviors were observed in Fluo-4-AM
 loaded myocytes that were deliberately not patch-clamped
 so as to leave the intracellular milieu minimally perturbed.
 Fig. 6 B shows $[\text{Ca}]_i$ fluorescence and a line scan from
 such a myocyte, taken during a long recording in which
 all activity was spontaneous (see Fig. S6 for the entire
 recording). Although voltage was not directly recorded,
 AP upstrokes, DADs, EADs, and full repolarization can
 be generally inferred from the patterns of Ca release
 observed in the line scan, because voltage-gated Ca release
 is reflected by vertical lines and Ca waves by chevrons.
 In this tracing, a Ca wave in the upper portion of the line
 scan (arrow) initiated a triggered AP, in which multiple sec-
 ondary Ca oscillations began immediately and grew in
 amplitude (reflecting EADs) before full repolarization.
 Repolarization was followed by two diastolic Ca waves
 (chevrons) that failed to trigger APs, after which a third
 Ca wave originating from the lower half of the line scan

FIGURE 5 Modulation of DADs by APD. (A) Voltage, $[\text{Ca}]_i$, and $[\text{Ca}]_{\text{SR}}$ versus time showing a regular AP followed by a small DAD. (B) Same as (A) but for a long AP with EADs followed by large DAD. (Insets) Line scans of $[\text{Ca}]_i$ during the DAD periods. (C-F) Plots of DAD amplitude versus APD (C), SR load (E), diastolic $[\text{Ca}]_i$ (F), and coupling interval versus APD (D) for DADs after APs of varying duration without (circle) or with (triangle) EADs. The definitions of SR load and dia-
 stolic $[\text{Ca}]_i$ (arrows) are indicated in (A) and (B). The coupling interval was defined as the interval from the end of the AP to the peak of the DAD.

The parameters are the same as in Fig. 1 A, but under
 Ca overload ($[\text{Ca}]_o = 5 \text{ mM}$) with $[\text{Na}]_i = 10 \text{ mM}$
 and 1 mV left-shift in $\Delta I_{\text{Ca,L}}$. The control $I_{\text{Ks}}$
 was used in (B) but was increased by a factor of
 1.6 to shorten APD in (A). To see this figure in color,
go online.
A triggered a second AP upstroke. Unlike the first AP, Ca oscillations during the second AP did not develop immediately, and the Ca release pattern was initially disordered, as evident from the line scan. The dysynchronous Ca release then gradually organized into synchronous oscillations with complex variations in amplitude. The largest release after the brief pause may reflect a late phase-3 EAD, because it did not appear to be triggered by a preceding Ca wave. After this event, the Ca oscillations became much smaller but quickly grew in amplitude, culminating in full repolarization, followed by a diastolic Ca wave (chevron) that failed to trigger an AP.

Several features are notable in both of these experimen-tal recordings. When an AP was preceded by a relatively long diastolic interval (as in Fig. 6 A and the second AP in Fig. 6 B), the initial Ca transient during the AP upstroke was followed by dysynchronous Ca release with only small fluctuations in the plateau voltage and whole cell Ca. With time, however, these fluctuations self-organized into synchronous oscillations that subsequently grew progressively in amplitude, eventually culminating in full repolarization. In contrast, after a short diastolic interval (Fig. 6 B, first and third APs), the early phase of dysynchronous Ca releases did not occur, and synchronous Ca oscillations began immediately and grew rapidly, shortening the time to full repolarization. The features of the EADs generally agree with observations in previous experiments (e.g., long plateau, complex EAD pattern, and EAD-DAD interactions), as illustrated in Figs. S2 and S3.
constant until EADs occurred. The relatively stable SR Ca content indicates that the increase in [Ca], resulted primarily from maintained Ca entry via $I_{Ca,L}$. As shown in the line scan, before the onset of frank EADs, Ca release exhibited a random spatiotemporal pattern—one that gradually became more synchronous as EADs developed. When the EADs reached a large enough amplitude, $I_{Ca,L}$ activation became sufficient to induce full repolarization. A second paced AP was then elicited after a short diastolic interval. Similar to the second AP in Fig. 6, the long plateau disappeared and EADs and Ca oscillations began immediately as the voltage decayed into the LCC window voltage range.

Because the experiments in Fig. 6 were done in mouse ventricular myocytes exposed to elevated extracellular [Ca], we also carried out computer simulations using a mouse ventricular myocyte model with the ionic currents from the model by Morotti et al. (38) (see the Supporting Materials and Methods). Similar complex EAD behaviors and spontaneous APs were obtained (see Fig. S6), indicating that the behaviors are not model-specific.

EADs and Ca oscillations with alternating or more complex patterns are shown in Fig. 8. In this example, both the take-off potential and amplitude of the EADs exhibited a period-3-like pattern most of the time. Moreover, switching between EADs and DAD-mediated-triggered APs (asterisks in Fig. 8) occurred. These behaviors are similar to Fig. 6 as well as previous experiments (sample voltage traces are shown in Fig. S3) (3,31,37). Note that in Fig. 8, the first AP was elicited by a stimulus and the following three APs were triggered by DADs. As shown in Fig. 5, lengthening of APD by EADs causes excessive Ca loading of the cell, promoting spontaneous Ca release after full repolarization, resulting in DADs. Thus, the long APs with many EADs enhanced Ca overload and thus promoted DADs, illustrating a case of complex EAD and DAD interactions. The underlying mechanisms are addressed in the Discussion.

**DISCUSSION**

EADs and DADs were first described more than a half century ago (39,40), and many EAD and DAD behaviors, from simple to very complex, have been observed in experiments. However, due to the complex interactions between voltage and Ca, it has been difficult to pinpoint experimentally the exact underlying mechanisms for different behaviors. Computer modeling and simulations are a complementary means to reveal the underlying ionic mechanisms. Many computer modeling studies (41–46) have been carried out to investigate the mechanisms of EADs, but for the most part these have focused on EADs caused by voltage-driven oscillations, because the AP models lacked detailed spatiotemporal Ca cycling required to simulate Ca waves and oscillations. In this study, we investigated the effects of bidirectional Ca-voltage coupling on the genesis of EADs and DADs in ventricular myocytes, using an AP model with a detailed spatiotemporal Ca cycling regulation incorporating stochastic LCC and RyR openings and experiments of mouse ventricular myocytes. Mechanistic insights we consider novel have been revealed, and these are discussed below.

**Modulations of EADs by Ca cycling dynamics**

It is well known that LCC window current is a key component for EADs (17–21), but all other currents during the plateau also contribute to the formation of the EADs (21,41). Using bifurcation analysis (21,44), we have previously identified the bifurcations that initiate and terminate the voltage oscillations, and characterized the contribution of each ionic current in generating these bifurcations. The Ca transient modulates EAD dynamics via its effects on multiple Ca-dependent ionic currents. Because the [Ca], dependences of these inward and outward currents are different, the net effect of changing [Ca], on the bifurcations and the time course of voltage oscillations is complex and often nonintuitive. Changing [Ca], can either promote or suppress EADs, depending on the balance of factors under a given set of conditions (Figs. 2 and 3).

Spontaneous Ca oscillations have been proposed to cause EADs in a number of experimental studies (10,11,13,28,29), primarily based on evidence such as a rise in [Ca], preceding the EAD upstroke (28), or continuation of spontaneous Ca oscillations when voltage oscillations were prevented by imposing an AP clamp (13). A fresh mechanistic insight...
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arising from our analysis is that the interpretation of these experimental findings may be overly simplistic. Intuitively, because voltage and Ca are coupled, if Ca oscillates in the repolarizing phase of the AP, voltage will also oscillate. However, based on our present simulations (Fig. 4), the role of spontaneous Ca oscillations as a pure mechanism for generating EADs is far from straightforward. First, \( \text{I}_{\text{Ca,L}} \) is not a regenerative current like \( \text{I}_{\text{Ca,L}} \). That is, as voltage depolarizes during the EAD upstroke, \( \text{I}_{\text{NCX}} \) becomes weaker, not stronger. Because \( \text{I}_{\text{NCX}} \) is small and decreases as voltage rises, reactivation of \( \text{I}_{\text{NCX}} \) is required for EADs to become large enough to generate TA and propagate in tissue. Thus, a spontaneous Ca release event can potentially initiate an EAD upstroke by activating \( \text{I}_{\text{NCX}} \) but cannot generate significant EAD amplitude or TA without the participation of \( \text{I}_{\text{Ca,L}} \) reactivation. This situation is further complicated by the interactions between intracellular Ca and \( \text{I}_{\text{Ca,L}} \) inactivation, which inevitably affect the \( \text{I}_{\text{Ca,L}} \) window current, the key driver for voltage-dependent EADs. As shown in Fig. 4, high intracellular Ca due to spontaneous Ca release just preceding the EAD strongly enhanced Ca-dependent inactivation of \( \text{I}_{\text{Ca,L}} \), which offset its effect at increasing \( \text{I}_{\text{NCX}} \). The consequence was a lower take-off potential of the EAD, which then resulted in a large-amplitude EAD due to greater \( \text{I}_{\text{Ca,L}} \) reactivation. However, additional EADs were suppressed due to a stronger Ca-induced inactivation of \( \text{I}_{\text{Ca,L}} \). This scenario may be relevant to isoproterenol-induced EADs, which typically exhibit only one EAD in each AP, with two or more EADs per AP being rare (10,11,13). In summary, the modeling results show that the effect of spontaneous Ca release events on EAD genesis is extremely complex, and intuitive interpretations, like assuming that the timing of the intracellular Ca rise relative to the EAD upstroke implies causality, are overly simplistic.

Mechanisms of DADs

It is widely accepted that DADs are mediated by Ca waves (47–50), in which spontaneous Sr Ca release from a group of adjacent CRUs recruits neighboring CRUs to initiate a propagating wave (51,52). On the other hand, the simulations in Fig. 5A demonstrate, to our knowledge, a novel mechanism of small DADs with long coupling intervals in the absence of Ca waves, purely by synchronous resetting of the recovery period of CRUs by an AP, without recruitment. In this case, after an AP with normal Ca release, the Sr Ca rose to a level higher than its equilibrium load. This occurred because the SERCA pump is fast relative to NCX, causing the SR to refill to a higher level. After the CRUs had recovered from the preceding normal AP, spontaneous CRU firings occurred due to high Sr Ca load, which summed to produce a small whole-cell [Ca], increase eliciting a small DAD. As seen in the line scans in Fig. 5A, the release events were random individual CRU firings that did not propagate to neighboring CRUs. In contrast, in Fig. 5B, the EADs during the AP greatly enhanced the cellular Ca load, elevating diastolic [Ca],. In this case, when CRUs fired during the subsequent diastole, they propagated as miniwaves, facilitated by both the high Sr Ca content and the elevated diastolic [Ca] level. Therefore, in the non-Ca-wave-mediated mechanism of DADs in Fig. 5A, the high Sr Ca load due to fast SERCA pump activity after an AP synchronizes the subsequent diastolic spontaneous Ca release by independently firing CRUs. However, diastolic [Ca] is not sufficiently elevated for these Ca sparks to trigger propagating Ca waves. For this reason, only a single DAD occurs after the AP. On the other hand, in the Ca-wave-mediated mechanism, diastolic [Ca], as well as Sr Ca load both become important by facilitating CRU recruitment to form Ca waves, and multiple DADs can be seen after an AP. Finally, although not addressed in this study, DAD amplitude is determined not only by the synchronicity of spontaneous Ca release, but also by the Ca-voltage coupling gain (53,54), i.e., the amplitude of the Ca-sensitive currents such as \( \text{I}_{\text{NCX}} \) and \( \text{I}_{\text{Ca,L}} \) activated by the spontaneous Ca transient in relation to the outward currents opposing depolarization, principally \( \text{I}_{\text{K}} \) in ventricular tissue.

Mechanisms of complex EAD patterns

As shown in Figs. 6, 7, 8, and 51–53, EADs can exhibit very complex patterns and behaviors. From this study and the nonlinear dynamics analysis (21,44), we can provide a unified theory for these complex EAD patterns based on the interactions of Ca dynamics with the Hopf-homoclinic bifurcation mechanism of voltage-driven EADs. Fig. 9 shows a schematic diagram illustrating the bifurcations leading to and terminating EADs. By 100–200 ms after the AP upstroke, most currents have reached quasi-steady states except for slow currents, including slowly activating \( \text{I}_{\text{K}} \), slowly inactivating late \( \text{I}_{\text{K}} \), slow accumulation of

![Schematic diagrams for the mechanisms of EADs. Schematic diagram showing the bifurcations for EADs. The value \( V_p \) is the quasi-equilibrium steady-state voltage in the plateau, and \( V_{\text{rest}} \) is the resting potential. To see this figure in color, go online.](image)
[Na], causing slowly changing \( I_{\text{NaK}} \), etc. The inward currents and the outward currents are roughly equal during the AP plateau, forming a quasi-equilibrium state. As time continues, slowly increasing outward currents progressively activate, destabilizing the quasi-equilibrium state and initiating oscillations via a Hopf bifurcation. As the net outward current at the trough of the EADs becomes progressively larger during the voltage oscillations, the system approaches another bifurcation point, called a homoclinic bifurcation, at which the oscillation terminates because the inward current is no longer strong enough to prevent the voltage from repolarizing, leading to full repolarization in the resting potential. Depending on the stability of the quasi-equilibrium state, the decay rate of voltage, and the activation speed of the slowly increasing net outward current, as well as Ca-voltage coupling, different EAD patterns can occur. Based on the bifurcation theory and results in previous simulations (21,44,55–57) and the observations in this study, we summarize below the mechanisms underlying six characteristic patterns of EAD behaviors:

**EADs with growing amplitude**

This is the most widely seen EAD pattern in experiments (Fig. S1 A) and computer simulations. As shown in Fig. 9, after the Hopf bifurcation point, the oscillation amplitude grows as the slowly increasing outward current makes the trough of the EAD more and more negative, reaching a maximum at the homoclinic bifurcation point. If voltage decays into the window voltage range at roughly the same time as the slowly increasing outward current increases to the level sufficient to initiate the Hopf bifurcation, then the EAD amplitude will grow from very small oscillations to the maximum oscillation at the homoclinic bifurcation point before full repolarization.

**EADs with decreasing amplitude (Fig. S1 B)**

Although the quasi-equilibrium state is stable before the Hopf bifurcation point, the voltage entering into the window range may spiral toward the quasi-equilibrium state (see the spiral curve in Fig. 9), resulting in a transiently decreasing oscillation amplitude. If the Hopf-homoclinic bifurcation does not exist, then the oscillation gradually damps out until repolarization to the resting potential occurs, resulting in EADs with decreasing amplitude. This scenario was analyzed in detail using bifurcation theory by Xie et al. (56).

**EADs with decreasing and then increasing amplitude (Fig. S2 A)**

If voltage decays into the window range earlier than the Hopf bifurcation, then the voltage may oscillate transiently with decreasing amplitude (see the spiral curve in Fig. 9). As the slowly increasing outward current increases to the critical value initiating the Hopf bifurcation, oscillation resumes with a growing amplitude, reaching its maximum at the homoclinic bifurcation point.

**EADs after a long plateau (Figs. 6, 7, and S2 A and B)**

If voltage decays to the plateau level much earlier than the Hopf bifurcation, it will remain at the plateau voltage until the slow outward current increases sufficiently to induce the Hopf bifurcation for oscillations. Therefore, the duration of the plateau depends on the speed of the slowly growing outward current (or slowly decreasing inward current). In the simulation in Fig. 7 using our AP model based on rabbit ventricle, \( I_{\text{Ks}} \) is the slowly increasing current, such that the onset of oscillations is very sensitive to \( I_{\text{Ks}} \) (Fig. 7): the plateau is shorter when \( I_{\text{Ks}} \) starts at a larger value (referenced by the dashed horizontal line in the \( I_{\text{Ks}} \) trace in Fig. 7), e.g., after a short diastolic interval.

A key question is why the intracellular Ca, despite being quite high, does not oscillate during the plateau phase until EADs are present. To reveal the underlying mechanisms, we did a series of simulations under different voltage-clamp conditions (see Figs. S7 and S8 and description). We showed that random LCC openings trigger spatiotemporally random CRU firings, which prevent the CRUs from recovering synchronously as required for spontaneous Ca oscillations. After the Hopf bifurcation, however, the voltage oscillations cause LCC openings to synchronize, which synchronizes the CRU openings, so that voltage and Ca oscillate together.

Although \( I_{\text{Ks}} \) played the major role as the slowly increasing outward current in our AP model based on rabbit ventricle, other slowly changing currents can also play the equivalent role, and may be more important in other species. For example, [Na], accumulates very slowly, causing a very slow increase in \( I_{\text{NaK}} \), which can also initiate the Hopf-homoclinic bifurcation, as shown in our previous simulations (57). Late \( I_{\text{Ks}} \) is another candidate because it may slowly inactivate (38). Finally, the gradual rise in [Ca], may also activate other outward currents, such as the Ca-activated Cl current (\( I_{\text{ClCa}} \)) (59) or small conductance Ca-activated K current (60). Because \( I_{\text{Ks}} \) has a very low density in mouse ventricular myocytes, one or more of these currents may be responsible for the slow process that is causing the long plateau shown in the experimental recordings in Fig. 6.

**EAD bursts (Fig. S2 C)**

If voltage quickly decays into window voltage for oscillations and then outward current increases very slowly through the Hopf-homoclinic bifurcation window, a very long EAD burst can result. As demonstrated in our previous simulations (57), the slow accumulation of [Na], causing a very slow increase of \( I_{\text{NaK}} \) can be a candidate for finally terminating the long-lasting EAD burst.

**EAD alternans and more complex EAD patterns (Figs. 6, 8, and S3)**

To our knowledge, a key novel finding of this study is that the Hopf-Homoclinic bifurcation dynamics alone do not explain...
alternating and more complex EAD patterns. As shown by the simulations in Figs. S7 and S8, EAD alternans arises as a result of Ca-voltage coupling when voltage oscillations during EADs encounter CRU refractoriness, generating Ca transient alternans or more complex patterns. The Ca transient behaviors then feed-back to affect the amplitude of the EADs via Ca-sensitive currents, or via frequency competition of voltage oscillation and spontaneous Ca oscillations. Therefore, EAD alternans and more complex EAD patterns directly result from bidirectional Ca-voltage coupling.

**Limitations**

Several limitations should be noted. The AP and spatiotemporal Ca cycling model in this study simulates a three-dimensional network of CRUs representing a ventricular myocardium, but it is still much simpler than a real myocardium (61,62). Heterogeneous ion channel densities and CRU properties may potentially introduce novel dynamics not captured in a homogeneous model. However, the homogeneous model appears to capture most of the experimentally observed behaviors, and in addition provides a key framework for future exploration of the role of heterogeneity. The RyR model developed by Restrepo et al. (14) incorporated calsequestrin-mediated SR luminal Ca regulation, whereas recent experiments have demonstrated a luminal Ca-sensing site in the RyR mediating this effect (63). Moreover, the refractoriness of Ca release is controversial, with a wide range of experimentally measured values (64–69) and different proposed causes. Different refractory period choices may impact the Ca cycling dynamics, as well as the voltage dynamics caused by Ca-voltage coupling. In our model, the effects of Ca on EADs are mediated through direct effects on ionic currents. It is well known that Ca-dependent signaling pathways, such as CaMKII signaling, can affect many ionic currents as well as Ca cycling properties (70), which can have much more complex effects on EADs and DADs. Our AP model does not include some of the Ca-dependent ionic currents, such as \( I_{CaL} \) (71,72) and the small conductance Ca-activated K channel (60), which may play important roles in bidirectional Ca-voltage coupling and Ca-dependent EAD genesis. In addition, we have clamped [Na\(_{\text{S}}\)] in this study. It has been shown that elevation of [Ca\(_{\text{S}}\)], causing activation of CaMKII enhances late \( I_{Ks} \), which further elevates [Na\(_{\text{S}}\)], causing further elevation of [Ca\(_{\text{S}}\)] (38). Therefore, a dynamic [Na\(_{\text{S}}\)] may have additional effects on EADs and DADs.

Another limitation is that the specific ionic mechanisms of EADs may vary among different species. For example, in the rabbit model, slow activation of \( I_{Ks} \) plays a key role in the EAD dynamics, whereas \( I_{Ks} \) is a very small current in mouse ventricular myocytes. As discussed earlier, however, \( I_{Ks} \) is not the only slow component that can play this role in the dynamics. Other candidates include slow Na accumulation activating outward \( I_{NaK} \), the progressive Ca accumulation activating \( I_{CaK+} \), and/or slow inactivation of late \( I_{Ks} \), all of which can in principle replace the role of \( I_{Ks} \) in initiating and terminating EADs via the Hopf-homoclinic bifurcation scenario. In the mouse ventricular cell model in which \( I_{Ks} \) is insignificant, the slow inactivation of late \( I_{Ks} \) substitutes for the role of \( I_{Ks} \) in the rabbit ventricular cell model (see Supporting Materials and Methods and Fig. S6). Because the complex EAD behaviors have been widely observed in many different types of cardiac cells and species exposed to varying pathophysiological stresses (e.g., Figs. 6 and S1–S3), we believe that different species and cell types share a common general dynamical mechanism, i.e., the Hopf-homoclinic bifurcation scenario depicted in Fig. 9. Our study focused mainly on phase-2 EADs and DADs, and did not address phase-3 EADs, which may involve additional or different mechanisms.

Finally, although our simulation results of complex EAD behaviors agree well with our own experimental observations and those from the literature, some of the theoretical predictions of our study need to be validated by future experiments. These would include the complex effects of the amplitude and morphology of Ca transient on modulating EADs, the effects of spontaneous Ca release on generating and suppressing EADs, and the non-Ca-wave-mediated DADs.

**SUPPORTING MATERIAL**

Supporting Materials and Methods, nine figures, and ten tables are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(15)00244-1.

**AUTHOR CONTRIBUTIONS**

Z.Q. designed the overall research and supervised the simulations; Z.S. contributed to the overall research design and performed the simulations; J.N.W. contributed to the overall research design and supervised the biological experiments; C.Y.K. contributed to the overall research design and performed the biological experiments; M.N. contributed to the overall design; Z.S., C.Y.K., J.N.W., and Z.Q. analyzed data; and Z.S., C.Y.K., J.N.W., and Z.Q. wrote the article.

**ACKNOWLEDGMENTS**

This work is supported by National Institutes of Health grants No. P01 HL078931, No. RO1 HL110791, and No. R56 HL118041, a research grant from Gilead Sciences (to Z.S.), and Laubisch and Kawata endowments.

**REFERENCES**

Cardiac Afterdepolarizations


Supplemental Methods

A. Myocyte experiments

**Myocyte Isolation.** Mouse ventricular myocytes were isolated enzymatically as described previously (2). Briefly, C57Bl/6 mice (6-8 weeks) were injected intraperitoneally with 800 µl heparin (5,000 units/ml) 20-30 minutes before anesthetization and sacrifice with isoflurane (Phoenix Pharmaceuticals, Inc.). Hearts were quickly excised by thoracotomy and retrogradely perfused on a Langendorff apparatus maintained at 37°C. Enzyme digestion step consisted of perfusing Tyrode’s solution containing 1 mg/ml collagenase (Type II; Worthington) and 2.8 mg/ml protease (Type XIV, Sigma) for 13-15 minutes. Myocytes were separated from digested ventricles by gentle mechanical dissociation and used within 4-6 hours. The modified Tyrode’s solution contained the following (mmol/L): 136 NaCl, 5.4 KCl, 0.33 NaH₂PO₄, 1.0 MgCl₂, 10 HEPES, and 10 glucose; pH 7.4 (KOH). All chemicals were purchased from Sigma unless indicated otherwise. All procedures comply with UCLA Animal Research Committee policies.

**Patch Clamp Recordings.** APs from isolated myocytes were recorded in the current clamp mode using the perforated patch technique with Amphotericin B (240 µg/ml), as previously described (6, 7). Borosilicate glass electrodes (tip resistance 1.5-3 MΩ) were filled with internal solution containing (in mmol/L) 110 K-Aspartate, 30 KCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 5 MgATP, 5 creatine phosphate, and 0.1 cAMP; pH 7.2 (KOH). The standard bath Tyrode’s solution contained (in mmol/L): 136 NaCl, 5.4 KCl, 0.33 NaH₂PO₄, 1.0 MgCl₂, 10 HEPES, 1.8 CaCl₂, and 10 glucose; pH 7.4 (KOH). Extracellular [Ca] was raised to 2.7 mmol/L to facilitate EADs and DADs. APs were elicited with square current pulses of 2 ms duration and twice threshold amplitude. Data were acquired with an Axopatch 200A patch-clamp amplifier and Digidata 1200 acquisition board driven by pCLAMP 9.0 software (Axon Instruments, Inc.). Corrections were made for liquid junction potentials. Signals were filtered at 1 kHz. All experiments were carried out at 37°C.

**Calcium Imaging.** Myocytes were incubated with fluorescent Ca indicator dye Fluo-4 AM (10 µM, Life Technologies) and nonionic surfactant Pluronic F-127 (0.02%, Life Technologies) in Tyrode’s solution for 30 minutes at room temperature before imaging. Ca fluorescence in isolated myocytes was recorded using an inverted Nikon Diaphot microscope (60X objective, Olympus) equipped with a charge-coupled device (CCD)-based Photometrics Cascade 128+ camera (~120 frames/s, 128 x 128 pixels) operating under Imaging Workbench software (version 6.0, INDEC BioSystems). Voltage was concurrently recorded in patch-clamped myocytes. Pseudo-line scan images were generated from the acquired video data using ImageJ software (11).

B. Rabbit ventricular cell model

1. The spatial structure of ventricular myocyte model

The ventricular myocyte model is a three-dimensional object containing 19,305 (65×27×11) CRUs (FIGURE) with CRU spacing being 1.84 µm in the longitudinal direction and 0.9 µm in the transverse direction, corresponding to a dimension 121 µm × 25 µm × 11 µm. The CRUs are coupled via Ca diffusion in the cytosolic space and SR. The model was modified from the one developed by Restrepo et al. (12). The details of the model are described in the sections below. Briefly, each CRU contains five sub-volumes with defined volume ratios (right panel in FIG.A): network SR (NSR), junctional SR (JSR), dyadic space (DS), sub-membrane space (SUB), and cytosolic space (CYTO). Ca from extracellular space enters into DS via LCCs and is released from the JSR to DS via RyRs. Each CRU has a cluster of 100 RyR channels associated with a cluster of 12 LCCs, both simulated using random Markov transitions. Ca is extruded from the SUB space via NCX and taken up into the NSR from CYTO via SERCA pump. Ca diffuses freely between the SR sub-volumes and between the cytosolic sub-volumes. CRUs are coupled via Ca diffusion between neighboring SR spaces, SUB.
spaces, and CYTO spaces, respectively. No Ca diffusion exists directly between neighboring JSR spaces or between neighboring DS spaces.

However, a directly randomized version of the HH-type formulation is not appropriate for an LCC because the maximum open probability of an LCC is 100% in the HH-type model at high voltages, whereas that of real channels is much smaller (~5%). To reduce the open probability, we added a new state (d3 in FIG.B) to the d gate of the HH-type model, with d1 being the activated state. The transitions rates \( \alpha_d, \beta_d, \alpha_f, \) and \( \beta_f \) are the same as in the Luo and Rudy model (13). The rate constants for Ca-dependent inactivation were modified to account for the fact that Ca concentration in the DS space, which is much higher than the bulk cytosolic Ca concentration, was used to mediate LCC inactivation. The steady state of d3 (the steady-state activation curve) is:

\[
d_{ss} = \frac{\alpha_d}{\alpha_d + \beta_d' (\alpha_d + \beta_d)}
\]
Since the open probability is small, i.e., \( \frac{\beta_d}{\alpha_d} >> 1 \), then \( d_{\infty} = \frac{\alpha_d}{\beta_d} \), which is a rescale of the original model. Therefore, by shifting the voltage dependence of \( \alpha_d \) and \( \beta_d \) or \( \alpha_f \) and \( \beta_f \), the window ICa,L can be changed directly without affecting other properties of the channel. The right panel of FIG.B shows the equivalent Markov scheme of the LCC of the modified HH-type model.

2. Voltage and ionic currents

The differential equation for membrane voltage is

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

where \( C_m = 1 \ \mu F/cm^2 \) is the cell membrane capacitance, \( I_{stim} \) is the stimulus current pulse which was set as -40 \( \mu A/cm^2 \) and duration 1 ms, and \( I_{ion} \) is the total membrane current density, which is described by

\[
I_{ion} = I_{Na} + I_{K1} + I_{Kr} + I_{Ks} + I_{Ko,f} + I_{Ko,s} + I_{NaK} + I_{Ca,L} + I_{NCX}
\]

The mathematical formulations of the ionic currents are detailed in following sub-sections and their conductance are listed in Table S1. The physical constants and ion concentrations are listed in Table S2.

### Table S1. Ionic current conductance

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g_{Na} )</td>
<td>( I_{Na} ) conductance</td>
<td>12.0 mS/( \mu F )</td>
</tr>
<tr>
<td>( g_{Ko,f} )</td>
<td>( I_{Ko,f} ) conductance</td>
<td>0.11 mS/( \mu F )</td>
</tr>
<tr>
<td>( g_{Ko,s} )</td>
<td>( I_{Ko,s} ) conductance</td>
<td>0.04 mS/( \mu F )</td>
</tr>
<tr>
<td>( g_{K1} )</td>
<td>( I_{K1} ) conductance</td>
<td>0.3 mS/( \mu F )</td>
</tr>
<tr>
<td>( g_{Kr} )</td>
<td>( I_{Kr} ) conductance</td>
<td>0.0125 mS/( \mu F )</td>
</tr>
<tr>
<td>( g_{Ks} )</td>
<td>( I_{Ks} ) conductance</td>
<td>0.1386 mS/( \mu F )</td>
</tr>
<tr>
<td>( g_{NaK} )</td>
<td>( I_{NaK} ) conductance</td>
<td>1.5 mS/( \mu F )</td>
</tr>
</tbody>
</table>

### Table S2. Physical constants and ionic concentrations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( F )</td>
<td>Faraday constant</td>
<td>96.5 C/mmol</td>
</tr>
<tr>
<td>( R )</td>
<td>Universal gas constant</td>
<td>8.315 J/mol·K⁻¹</td>
</tr>
<tr>
<td>( T )</td>
<td>Temperature</td>
<td>308 K</td>
</tr>
<tr>
<td>([Na]_o)</td>
<td>External sodium concentration</td>
<td>136 mM</td>
</tr>
<tr>
<td>([K]_o)</td>
<td>External potassium concentration</td>
<td>5.4 mM</td>
</tr>
<tr>
<td>([K]_i)</td>
<td>Internal potassium concentration</td>
<td>140 mM</td>
</tr>
<tr>
<td>([Ca]_o)</td>
<td>External calcium concentration</td>
<td>1.8 mM</td>
</tr>
</tbody>
</table>

2.1. Sodium current (\( I_{Na} \))

\[
I_{Na} = g_{Na} m^3 h (V - E_{Na}),
\]

\[
E_{Na} = \frac{RT}{F} \ln \left( \frac{[Na]_o}{[Na]_i} \right),
\]

\[
\frac{dh}{dt} = \alpha_h (1 - h) - \beta_h h,
\]

\[
\frac{dj}{dt} = \alpha_j (1 - j) - \beta_j j,
\]

\[
\frac{dm}{dt} = \alpha_m (1 - m) - \beta_m m,
\]
\[ \alpha_m = 0.32 \frac{V + 47.13}{1 - e^{-0.11(V + 47.13)}}, \]
\[ \beta_m = 0.08 e^{-0.1(V + 47.13)}. \]

For \( V \geq -40 \text{ mV} \),
\[ \begin{align*}
\alpha_h &= 0, \\
\alpha_j &= 0, \\
\beta_h &= \frac{0.13}{1 + e^{-11.1(V+10.66)}}, \\
\beta_j &= 0.3 e^{-2.535 \times 10^{-7}V}. 
\end{align*} \]

For \( V \leq -40 \text{ mV} \),
\[ \begin{align*}
\alpha_h &= 0.135 e^{-\frac{0.8V}{6.8}}, \\
\beta_h &= 3.56 e^{0.07W} + 3.1 \times 10^{-5} e^{0.3V}, \\
\alpha_j &= \frac{(-127140 e^{0.2444V} - 0.03474 e^{0.04391V}) \times (V + 37.78)}{1 + e^{0.311(V+79.23)}}, \\
\beta_j &= \frac{0.1212 e^{-0.01052V}}{1 + e^{-0.1378V(V+40.14)}}. 
\end{align*} \]

2.2. L-type Ca channel model and whole-cell \( I_{Ca,L} \)

The Ca channel model was modified as described in detail in Section 1. The opening of individual LCCs is simulated by a stochastic 12-state Markov model (panel B in FIGURE). Each CRU is assumed to have \( N_l \) LCCs under control condition. The Ca flux into the proximal space (dyadic space) of a CRU (the \((m, n, k)\)th CRU in cell) is given by
\[ I_{Ca,L}(m,n,k) = i_{Ca,L}(m,n,k)L(m,n,k), \]
where \( L \leq N_l \) is the number of open LCCs in the CRU, and \( i_{Ca,L} \) is the single channel current of the CRU which is:
\[ i_{Ca,L}(m,n,k) = \frac{4P_{Ca}zF(Y_1 c_p(m,n,k) e^{2z} - \gamma_0 \langle Ca \rangle_0)}{V^T e^{2z} - 1}. \]
\[ z = \frac{VT}{R^2}. \]
c\(_p\)\((m,n,k)\) is the Ca concentration in the corresponding proximal space of the CRU. Therefore, the whole-cell L-type Ca current \( I_{Ca,L} \) is summation of the Ca currents of CRUs in the cell, i.e.,
\[ I_{Ca,L} = \sum_{m,n,k=1}^{M,N,K} I_{Ca,L}(m,n,k) \]
where \( M, N, \) and \( K \) are the dimensions of the CRU network forming the cell.

The transition rates between different states of the LCC model are:
\[ \begin{align*}
\alpha_d &= \frac{d_{so}}{\tau_d}, \\
\beta_d &= \frac{1 - d_{so}}{\tau_d},
\end{align*} \]
where
\[ d_{so} = \frac{1}{1 + e^{\frac{V-5}{0.035(V-5)}},} \]
\[ \tau_d = \frac{1 - e^{-2.535 \times 10^{-7}V}}{0.035(V-5)} d_{so}. \]
\[
\alpha_f = \frac{f_\infty}{\tau_f}, \quad \beta_f = \frac{1-f_\infty}{\tau_f}
\]

where
\[
f_\infty = \frac{1}{1+e^{-\frac{V+32.06}{0.066}}},
\]
\[
\tau_f = \frac{0.0197e^{-0.0337(V+7)}+0.02}{\frac{c_\infty}{c_p}}.
\]
\[
\alpha_{f_{Ca}} = \frac{f_{Ca\infty}}{\tau_{f_{Ca}}}, \quad \beta_{f_{Ca}} = \frac{1-f_{Ca\infty}}{\tau_{f_{Ca}}}
\]

where
\[
f_{Ca\infty} = \frac{1}{1+\frac{c_{Ca\infty}}{c_p}}.
\]

\(\alpha_{d_2}\) and \(\beta_{d_3}\) are constants (Table S3). The parameters are listed in Table S3.

### Table S3. L-type Ca current parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(P_{Ca})</td>
<td>L-type channel permeability</td>
<td>11.9 (\mu\text{molC}^{-1}\text{ms}^{-1})</td>
</tr>
<tr>
<td>(\gamma_1, \gamma_0)</td>
<td>Activity coefficient of Ca</td>
<td>0.341</td>
</tr>
<tr>
<td>(N_L)</td>
<td>Number of LCCs in each CRU</td>
<td>12</td>
</tr>
<tr>
<td>(\bar{c}_P)</td>
<td>Threshold of Ca-dependent inactivation</td>
<td>6 (\mu\text{M})</td>
</tr>
<tr>
<td>(\tau_{f_{Ca}})</td>
<td>Time constant of Ca-dependent inactivation</td>
<td>15 ms</td>
</tr>
<tr>
<td>(\alpha_{d_2})</td>
<td>Transition rate from (d_2) to (d_3)</td>
<td>0.3 ms(^{-1})</td>
</tr>
<tr>
<td>(\beta_{d_3})</td>
<td>Transition rate from (d_3) to (d_2)</td>
<td>6 ms(^{-1})</td>
</tr>
</tbody>
</table>

#### 2.3. Sodium-calcium exchange current \((I_{NCX})\)

The Na-Ca exchange are spatially distributed in the CRUs, which are functions of Ca concentrations of the local sub-membrane spaces \((c_i)\). For the \((m,n,k)^\text{th}\) CRU, the Na-Ca change current is:

\[
I_{NCX}(m,n,k) = \frac{K_a \gamma_{NaCa} \left(e^{\eta [Na_i][Ca_i]} - e^{-\eta [Na_i]} \right)}{(t_1 + t_2 + t_3) (1+k_{sat} e^{(\eta - 1)z})},
\]

where
\[
t_1 = K_{mCa} [Na]_3^2 \left[1 + \frac{[Na_i]}{K_{mNa_i}}\right]^3,
\]
\[
t_2 = K_{mNa} G_3 (m,n,k) (1 + \frac{c_S(m,n,k)}{K_{mCa}})^3
\]
\[
t_3 = K_{mCa}^3 [Na]_3^2 + [Na]_3^2 [Ca_i] + [Na]_3^2 c_S(m,n,k),
\]
\[
K_a = \left[1 + \frac{K_{da}}{c_S(n,m,k)}\right]^{3-1},
\]
\[
z = \frac{VF}{RT}
\]

and the whole-cell \(I_{NCX}\) is:
\[ I_{NCX} = \sum_{m,n,k=1}^{M,N,K} I_{NCX}(m, n, k) \]

The parameters are listed in Table S4.

### Table S4. Sodium-calcium exchange current parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>( v_{NaCa} )</td>
<td>?</td>
<td>( \mu \text{M} \ ms^{-1} )</td>
</tr>
<tr>
<td>( K_{mCa} )</td>
<td>3.59</td>
<td>( \mu \text{M} )</td>
</tr>
<tr>
<td>( K_{mCao} )</td>
<td>1.3</td>
<td>( \text{mM} )</td>
</tr>
<tr>
<td>( K_{mNai} )</td>
<td>12.3</td>
<td>( \text{mM} )</td>
</tr>
<tr>
<td>( K_{mNao} )</td>
<td>87.5</td>
<td>( \text{mM} )</td>
</tr>
<tr>
<td>( K_{da} )</td>
<td>0.11</td>
<td>( \mu \text{M} )</td>
</tr>
<tr>
<td>( k_{sat} )</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>( \eta )</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.4. Inward rectifier K current (\( I_{K1} \))

\[ I_{K1} = g_{K1} \sqrt{\frac{[K]_0}{5.4}} \frac{A_{K1}}{A_{K1} + B_{K1}}(V - E_K), \]

\[ A_{K1} = \frac{1.02}{1 + e^{0.2385(V - E_K - 59.2157)}}, \]

\[ B_{K1} = \frac{0.49124e^{0.08032(V - E_K + 5.476)} + e^{0.06175(V - E_K - 59.3143)}}{1 + e^{-0.5143(V - E_K + 4.753)}}, \]

\[ E_K = \frac{RT}{F} \ln \left( \frac{[K]_0}{[K]_I} \right). \]

#### 2.5. The rapid component of the delayed rectifier K current (\( I_{Kr} \))

\[ I_{Kr} = g_{Kr} \sqrt{\frac{[K]_0}{5.4}} x_{Kr} R(V)(V - E_K), \]

\[ R(V) = \frac{1}{1 + e^{3.33}}, \]

\[ \frac{dx_{Kr}}{dt} = \frac{x_{Kr} - x_{Kr}}{\tau_{Kr}}, \]
2.6. The slow component of the delayed rectifier K current (I_{KS})

\[ I_{KS} = g_{KS} x_1 x_2 q_{KS} (V - E_{KS}), \]
\[ q_{KS} = \frac{1}{1 + \left(\frac{0.5}{c_i}\right)^3}, \]
\[ \frac{dx_1}{dt} = \frac{x_2^\infty - x_1}{\tau_{xs1}}, \]
\[ \frac{dx_2}{dt} = \frac{x_2^\infty - x_2}{\tau_{xs2}}, \]
\[ x_1^\infty = \frac{1}{1 + e^{-\frac{V - 1.5}{16.7}}}, \]
\[ \tau_{xs1} = \frac{1}{0.0000719(V + 30) + 0.00031(V + 30) + 1 - e^{-0.0687(V + 30)}}, \]
\[ \tau_{xs2} = 4\tau_{xs1}, \]
\[ E_{KS} = \frac{RT}{F} \ln \left( \frac{\left[K\right]_o + 0.01833[Na]_o}{\left[K\right]_i + 0.01833[Na]_i} \right) \]

2.7. The fast component of the outward K current (I_{to,f})

\[ I_{to,f} = g_{to,f} X_{to,f} Y_{to,f} (V - E_{K}), \]
\[ X_{to,f}^\infty = \frac{1}{1 + e^{-\frac{V + 7}{15}}}, \]
\[ Y_{to,f}^\infty = \frac{1}{1 + e^{-\frac{V + 33.5}{10}}}, \]
\[ \tau_{Xto,f} = 3.5e^{-\left(\frac{V}{30}\right)^2} + 1.5, \]
\[ \tau_{Yto,f} = \frac{20}{1 + e^{-\frac{V + 33.5}{10}}} + 20. \]
\[ \frac{dX_{to,f}}{dt} = \frac{X_{to,f}^\infty - X_{to,f}}{\tau_{Xto,f}}, \]
\[ \frac{dY_{to,f}}{dt} = \frac{Y_{to,f}^\infty - Y_{to,f}}{\tau_{Yto,f}}. \]

2.8. The slow component of the outward K current (\(I_{to,s}\))

\[ I_{to,s} = g_{to,s}X_{to,s}(Y_{to,s} + 0.5R_s^\infty)(V - E_K), \]
\[ R_s^\infty = \frac{1}{1 + e^{\frac{V + 33.5}{10}}}, \]
\[ X_{to,s}^\infty = \frac{1}{1 + e^{\frac{V + 3}{15}}}, \]
\[ Y_{to,s}^\infty = \frac{1}{1 + e^{\frac{(V + 33.5)}{10}}}, \]
\[ \tau_{Xto,s} = \frac{9}{1 + e^{\frac{V + 3}{15}}}, \]
\[ \tau_{Yto,s} = \frac{3000}{1 + e^{\frac{V + 60}{10}}} + 30, \]
\[ \frac{dX_{to,s}}{dt} = \frac{X_{to,s}^\infty - X_{to,s}}{\tau_{Xto,s}}, \]
\[ \frac{dY_{to,s}}{dt} = \frac{Y_{to,s}^\infty - Y_{to,s}}{\tau_{Yto,s}}. \]

2.9. Sodium-potassium pump current (\(I_{NaK}\))

\[ \sigma = \frac{[Na]_o - 1}{7}, \]
\[ f_{NaK} = \frac{1}{1 + 0.1245e^{-\frac{V_F}{RT}} + 0.0365e^{-\frac{V_F}{RT}}}, \]
\[ I_{NaK} = g_{NaK}f_{NaK} \frac{1}{1 + \frac{[K]_o}{[Na]_i}} \times \frac{[K]_o}{[K]_o + 1.5}. \]
3. Intracellular Ca Cycling

3.1. Differential equations for Ca cycling

The Ca cycling are described by the following differential equations [for an arbitrary CRU at a location \((m,n,k)\) with the spatial location omitted in the equations]:

\[
\frac{dc_i}{dt} = \beta_i(c_i) \left( I_{d_{si}} v_i - I_{up} + I_{leak} - I_{TCi} + I_{ci} \right),
\]

\[
\frac{dc_s}{dt} = \beta_s(c_s) \left( I_{d_{ps}} v_s + I_{NCX} - I_{d_{si}} - I_{TCs} + I_{cs} \right),
\]

\[
\frac{dc_p}{dt} = \beta_p(c_p) \left( I_{rel} + I_{Ca,L} - I_{d_{ps}} \right),
\]

\[
\frac{dc_{n_{sr}}}{dt} = \left( I_{up} - I_{leak} \right) \frac{v_i}{v_{n_{sr}}} - \frac{v_{j_{sr}}}{v_{n_{sr}}} + I_{c_{n_{sr}}},
\]

\[
\frac{dc_{j_{sr}}}{dt} = \beta_j(c_{j_{sr}}) \left( I_{tr} - I_{r} \right) \frac{v_p}{v_{j_{sr}}},
\]

where \(c_i\) is the free Ca concentration in the bulk myoplasm, \(c_s\) is the free Ca concentration in the proximal space (dyadic space), \(c_{j_{sr}}\) is the free Ca concentration in the junctional SR, \(c_{n_{sr}}\) is the free Ca concentration in the network SR, \(\beta\) terms account for instantaneous buffer in corresponding compartments using the rapid buffering approximation, \(I_{up}\) is the SERCA uptake current representing total flux into the NSR, \(I_{leak}\) is the leak current from NSR to cytosol, \(I_{NCX}\) is Na-Ca exchange current, \(I_{Ca,L}\) is the L-type Ca influx, \(I_{rel}\) is the total Ca efflux from the JSR, \(I_{d_{si}}, I_{d_{ps}}\) and \(I_{d_{ps}}\) are the diffusion currents from adjacent compartments, \(I_{TCi}\) and \(I_{TCs}\) are the troponin C dynamic buffering currents in cytosol and submembrane spaces, \(I_{c_{n_{sr}}}\) and \(I_{c_{n_{sr}}}\) are the diffusive currents between neighboring CRUs in the corresponding compartments. Note that currents are all local currents for a single CRU, for example, \(I_{Ca,L}(m,n,k)\) and \(I_{NCX}(m,n,k)\) in the expressions of the whole-cell currents described in the sections of ionic currents. The values of compartments volumes are listed in Table S5.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(v_i)</td>
<td>Cytosolic volume</td>
<td>0.5 (\mu m^3)</td>
</tr>
<tr>
<td>(v_s)</td>
<td>Submembrane space volume</td>
<td>0.025 (\mu m^3)</td>
</tr>
<tr>
<td>(v_p)</td>
<td>Proximal space volume</td>
<td>0.00126 (\mu m^3)</td>
</tr>
<tr>
<td>(v_{j_{sr}})</td>
<td>JSR volume</td>
<td>0.02 (\mu m^3)</td>
</tr>
<tr>
<td>(v_{n_{sr}})</td>
<td>NSR volume</td>
<td>0.025 (\mu m^3)</td>
</tr>
</tbody>
</table>

3.2. Instantaneous cytosolic buffering

The factors \(\beta_i(c_i)\) and \(\beta_s(c_s)\) describe instantaneous buffering to Calmodulin, SR sites, Myosin \((Ca^{2+})\), and Myosin \((Mg^{2+})\). Note that the concentration of the proximal space rapidly equilibrates, so we do not require knowledge of the instantaneous buffers in the proximal space. The equation of \(\beta_i(c_i)\) is

\[
\beta_i(c_i) = \left[ 1 + \frac{\sum B_p K_p}{(c_i + K_p)^2} \right]^{-1},
\]

where the sum is over the instantaneous cytosolic buffers Calmodulin, SR sites, Myosin \((Ca^{2+})\), and Myosin \((Mg^{2+})\), with buffer dissociation constants \(K_{CAM}\), \(K_{SR}\), \(K_{MCA}\), and \(K_{MMG}\) and total concentration of buffering sites \(B_{CAM}\), \(B_{SR}\), \(B_{MCA}\), and \(B_{MMG}\), respectively. In addition to Calmodulin, instantaneous buffering in the submembrane space includes the subsarcolemmal sites of high affinity with total...
concentration of sites and dissociation constant $B_{SLH}$ and $K_{SLH}$, respectively. The parameters for instantaneous cytosolic buffering are in Table S6.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{CAM}$</td>
<td>7.0</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$B_{CAM}$</td>
<td>24.0</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$K_{SR}$</td>
<td>0.6</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$B_{SR}$</td>
<td>47.0</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$K_{M\text{Ca}}$</td>
<td>0.033</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$B_{M\text{Ca}}$</td>
<td>140.0</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$K_{SLH}$</td>
<td>0.3</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$B_{SLH}$</td>
<td>13.4</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$B_T$</td>
<td>70.0</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>$k_{\text{on}}^{T}$</td>
<td>0.0327</td>
<td>($\mu M$ ms)$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{off}}^{T}$</td>
<td>0.0196</td>
<td>(ms)$^{-1}$</td>
</tr>
</tbody>
</table>

### 3.3. Troponin C buffering

$I_{TCi}$ and $I_{TCS}$ describe the rate of change in the concentration of Ca bound to Troponin C in the cytosolic and submembrane compartments, $[CaT]_i$ and $[CaT]_s$. These quantities satisfy

$$\frac{d[CaT]}{dt} = I_{TCi},$$

with

$$I_{TCi} = k_{\text{on}}^{T}c_i(B_T - [CaT]_i) - k_{\text{off}}^{T}[CaT]_i,$$

and analogous expressions apply for the submembrane compartments, replacing the subscript $i$ by $s$. Here, $k_{\text{on}}^{T}$ and $k_{\text{off}}^{T}$ are the on- and off-rate constants for Ca Troponin C binding, and $B_T$ is the total concentration of Troponin C buffering sites. The values of these parameters are listed in Table S6.

### 3.4. Instantaneous luminal buffering

$B_{j\text{sr}}(c_{j\text{sr}})$ describes instantaneous luminal Ca buffering to calsequestrin (CSQN). The expression of $\beta_{j\text{sr}}(c_{j\text{sr}})$ is

$$\beta(c) = \left(1 + \frac{K_{B\text{CSQN}}n(c) + \partial_n(c)(cK_c + c^2)}{(K_c + c)^2}\right)^{-1},$$

where

$$n(c_{j\text{sr}}) = \frac{Mn_M + (1 - M)n_B}{B_{j\text{sr}}}$$

and

$$\bar{\rho} = \frac{1 + 8\rho B_{\text{CSQN}}}{4\rho B_{\text{CSQN}}} \frac{1}{(K^h + c_{j\text{sr}})^2},$$

and

$$\rho(c_{j\text{sr}}) = \frac{\rho_{\text{on}} c_{j\text{sr}}^h}{K^h + c_{j\text{sr}}^h}. $$

The parameters for luminal buffering are in Table S7.
3.5. SR Ca uptake current \((I_{\text{up}})\)

\[
I_{\text{up}} = v_{\text{up}} \left( \frac{(c_i/K')^H - (c_{\text{NSR}}/K_{\text{NSR}})^R}{1 + (c_i/K')^H + (c_{\text{NSR}}/K_{\text{NSR}})^R} \right).
\]

The parameters are listed in Table S8.

Table S7: Luminal buffering parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_{\text{CSQN}})</td>
<td>Concentration of CSQN molecules</td>
<td>460 (\mu)M</td>
</tr>
<tr>
<td>(K_C)</td>
<td>Dissociation constant of CSQN</td>
<td>600 (\mu)M</td>
</tr>
<tr>
<td>(n_M)</td>
<td>Buffering capacity of CSQN monomers</td>
<td>15</td>
</tr>
<tr>
<td>(n_D)</td>
<td>Buffering capacity of CSQN dimers</td>
<td>35</td>
</tr>
<tr>
<td>(\rho^-)</td>
<td>Asymptotic ratio of dimers to monomers</td>
<td>5000</td>
</tr>
<tr>
<td>(K)</td>
<td>Dimerization constant</td>
<td>850 (\mu)M</td>
</tr>
<tr>
<td>(H)</td>
<td>Dimerization exponent (steep)</td>
<td>23</td>
</tr>
</tbody>
</table>

3.6. SR leak current \((I_{\text{leak}})\)

\[
I_{\text{leak}} = g_{\text{leak}} \left( \frac{c_i^2}{c_{\text{JSR}}^2 + K_{\text{JSR}}^2} \right) (c_{\text{NSR}} - c_i).
\]

3.7. RyR release flux \((I_{\text{rel}})\)

\[
I_{\text{rel}} = J_{\text{max}} \frac{c_{\text{JSR}} - c_p}{v_p},
\]

where \(P_o\) is the fraction of RyR channels that are in the open state of RyRs. \(J_{\text{max}}\) is the maximum RyR flux strength. The parameters are listed in Table S9.

3.8. RyR model

The RyR model includes the following 4 states (Panel D in FIGURE): closed CSQN-unbound (CU), open CSQN-unbound (OU), open CSQN-bound (OB), and closed CSQN-bound (CB). The rates of transition are:

\[
k_{12} = K_u c_p^2,
\]
\[ k_{14} = \frac{\bar{\alpha} B_{\text{CSQN}}}{B^0_{\text{CSQN}}}, \]
\[ k_{21} = \tau_c^{-1}, \]
\[ k_{23} = \frac{\bar{\alpha} B_{\text{CSQN}}}{B^0_{\text{CSQN}}}, \]
\[ k_{32} = k_{41} k_{12} k_{43}. \]

The parameters are listed in Table S9. Note that \( B_{\text{CSQN}}/B^0_{\text{CSQN}} \) is only different from 1 when the CSQN concentration is modified.

### Table S9. SR release current and RyR model parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( J_{\text{max}} )</td>
<td>Maximum RyR flux strength</td>
<td>( 1.47 \times 10^{-2} \mu m^3 ms^{-1} )</td>
</tr>
<tr>
<td>( K_u )</td>
<td>CSQN-unbound opening rate</td>
<td>( 3.8 \times 10^{-4} \mu M^{-2} ms^{-1} )</td>
</tr>
<tr>
<td>( K_b )</td>
<td>CSQN-bound opening rate</td>
<td>( 1 \times 10^{-5} \mu M^{-2} ms^{-1} )</td>
</tr>
<tr>
<td>( \tau_u )</td>
<td>CSQN-unbinding timescale</td>
<td>700.0 ms</td>
</tr>
<tr>
<td>( \tau_b )</td>
<td>CSQN-binding timescale</td>
<td>10.0 ms</td>
</tr>
<tr>
<td>( \tau_c )</td>
<td>RyR closing timescale</td>
<td>1.0 ms</td>
</tr>
<tr>
<td>( B^0_{\text{CSQN}} )</td>
<td>Normal CSQN concentration</td>
<td>460 \mu M</td>
</tr>
</tbody>
</table>

### 3.9. Nearest-neighbor diffusive currents

The diffusive currents in cytosol, submembrane and NSR are given by

\[ I^{(n)}_{ci} = \sum \left( \frac{c^{(m)}_i - c^{(n)}_i}{\tau_{mn}} \right), \]

where the sum is over the six nearest neighbors. The values of the timescales (\( \tau_{mn} \)) are listed in Table S10.

### Table S10: Parameters of Ca Diffusion

<table>
<thead>
<tr>
<th>Parameter ( \tau )</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \tau_{\text{cy}} )</td>
<td>Transverse cytosolic</td>
<td>2.93 ms</td>
</tr>
<tr>
<td>( \tau_{\text{ly}} )</td>
<td>Longitudinal cytosolic</td>
<td>2.32 ms</td>
</tr>
<tr>
<td>( \tau_{\text{nrr}} )</td>
<td>Transversal NSR</td>
<td>7.2 ms</td>
</tr>
<tr>
<td>( \tau_{\text{nrl}} )</td>
<td>Longitudinal NSR</td>
<td>24.0 ms</td>
</tr>
<tr>
<td>( \tau_{\text{sl}} )</td>
<td>Transversal submembrane</td>
<td>1.42 ms</td>
</tr>
<tr>
<td>( \tau_{\text{sl}} )</td>
<td>Longitudinal submembrane</td>
<td>3.4 ms</td>
</tr>
</tbody>
</table>

### C. Mouse ventricular cell model

We used the same spatial CRU structure and distribution as the rabbit ventricular cell model described above but substituted the ionic currents of mouse ventricular myocytes formulated by Morotti et al (5) except \( I_{\text{Ca,L}} \) which was the same as in the rabbit ventricular cell model but a 65% increase in conductance, i.e.,
The details of the ionic currents are described below. The parameters are the same as in the Morotti et al unless specified.

**Sodium current (I\textsubscript{Na})**

\[
I_{Na} = g_{Na} m^3 h j (V - E_{Na}),
\]

\[
E_{Na} = \frac{RT}{F} \ln \left( \frac{[Na]_o}{[Na]_i} \right),
\]

\[
\frac{dh}{dt} = \alpha_h (1 - h) - \beta_h h,
\]

\[
\frac{dj}{dt} = \alpha_j (1 - j) - \beta_j j,
\]

\[
\frac{dm}{dt} = \alpha_m (1 - m) - \beta_m m,
\]

\[
\alpha_m = 0.32 \frac{V + 47.13}{1 - e^{-0.1(V + 47.13)}},
\]

\[
\beta_m = 0.08 e^{-\frac{V}{11}},
\]

For \( V \geq -40 \text{ mV} \),

\[
\alpha_h = 0,
\]

\[
\alpha_j = 0,
\]

\[
\beta_h = \frac{0.66}{0.13 \left( 1 + e^{-\frac{V + 10.66}{11.1}} \right)},
\]

\[
\beta_j = 0.3 \frac{e^{-2.535 \times 10^{-7} V}}{1 + e^{-0.3(V + 32)}},
\]

For \( V \leq -40 \text{ mV} \),

\[
\alpha_h = 0.135 e^{-\frac{V + 88}{32}},
\]

\[
\beta_h = 3.92 e^{0.079(V - 2)} + 3.1 \times 10^5 e^{0.35(V - 2)},
\]

\[
\alpha_j = \frac{(-127140 e^{0.2444V} - 0.03474 e^{-0.04391V}) \times (V + 37.78)}{1 + e^{0.311(V + 79.23)}},
\]
\[ \beta_j = \frac{0.1212 e^{-0.01052V}}{1 + e^{-0.1378(V+40.14)}}. \]

**Late sodium current (\(I_{Na,L}\))**

\[ I_{Na,L} = g_{Na,L} m_L^3 h_L (V - E_{Na,L}), \]

\[ E_{Na,L} = \frac{RT}{F} \ln \left( \frac{[Na]_o}{[Na]_i} \right), \]

\[ h_{L,0} = \frac{1}{1 + \exp \left( \frac{V + 91}{6.1} \right)} \]

\[ \tau_h = 4000 \text{ ms} \]

where \(m_L\) is the same as \(m\) in \(I_{Na}\).

**Background sodium current (\(I_{Na,bk}\))**

\[ I_{Na,bk} = g_{nbk} (V - E_{Na}) \]

**Inward rectifier potassium current (\(I_{K1}\))**

\[ I_{K1} = g_{K1} \sqrt{\frac{[K]_o}{5.4}} \frac{A_{K1}}{A_{K1} + B_{K1}} (V - E_K), \]

\[ A_{K1} = \frac{1.02}{1 + e^{0.2305(V-E_K-59.215)}}, \]

\[ B_{K1} = \frac{0.49124 e^{0.08032(V-E_K+5.476)} + e^{0.06175(V-E_K+594.31)}}{1 + e^{-0.5143(V-E_K+4.753)}}, \]

\[ E_K = \frac{RT}{F} \ln \left( \frac{[K]_o}{[K]_i} \right). \]

**The rapid component of the delayed rectifier potassium current (\(I_{Kr}\))**

\[ I_{Kr} = g_{Kr} \sqrt{\frac{[K]_o}{5.4}} x_{Kr} R(V) (V - E_K), \]

\[ R(V) = \frac{1}{1 + e^{72.4(V+33)}}, \]
\[
\frac{dx_{Kr}}{dt} = \frac{x_{Kr}^{\infty} - x_{Kr}}{\tau_{Kr}},
\]
\[
x_{Kr}^{\infty} = \frac{1}{1 + e^{-\frac{V+7}{7}}},
\]
\[
\tau_{Kr} = \frac{1}{\frac{0.00138(V+7)}{1 - e^{-0.123(V+7)}} + \frac{0.00061(V+10)}{1 + e^{0.145(V+10)}}}.
\]

The fast component of the transient outward potassium current (\(I_{to,f}\))

\[
I_{to,f} = g_{to,f}x_{to,f}y_{to,f}(V - E_K),
\]
\[
x_{to,f}^{\infty} = \frac{1}{1 + e^{-\frac{V+7}{33}}},
\]
\[
y_{to,f}^{\infty} = \frac{1}{1 + e^{-\frac{V+48}{5}}},
\]
\[
\tau_{X_{to,f}} = 0.7e^{-\frac{(V+25)^2}{30}} + 0.08,
\]
\[
\tau_{Y_{to,f}} = \frac{8}{1 + e^{-\frac{V+60}{8}}} + 32e^{-\left(\frac{V+55}{16}\right)^2} + 10,
\]
\[
\frac{dX_{to,f}}{dt} = \frac{x_{to,f}^{\infty} - X_{to,f}}{\tau_{X_{to,f}}},
\]
\[
\frac{dY_{to,f}}{dt} = \frac{y_{to,f}^{\infty} - Y_{to,f}}{\tau_{Y_{to,f}}},
\]

Sodium-potassium pump current (\(I_{NaK}\))

\[
\sigma = e^{-\frac{\sigma_0}{7}} - 1,
\]
\[
f_{NaK} = \frac{1}{1 + 0.1245e^{-0.31V_F \frac{\sigma}{RT}} + 0.0365\sigma e^{-0.31V_F \frac{\sigma}{RT}}},
\]
\[
I_{NaK} = g_{NaK}f_{NaK} \frac{1}{1 + \frac{19}{[Na_i]^0}} \times [K]^0_{o} + 1.5.
\]

L-type calcium current (\(I_{Ca,L}\))
The ICa,L formulation is the same as in the rabbit ventricular cell model except that the activation kinetics is shifted to the negative voltage direction for 15 mV, i.e.,

\[
d_\omega = \frac{1}{1 + e^{-\frac{V+10}{6.24}}},
\]

\[
\tau_d = \frac{1 - e^{-\frac{V+10}{6.24}}}{\tau_d(0.035(V + 10))} d_\omega.
\]

**Background calcium leak current (ICabk)**

\[I_{Ca_bk} = g_{Ca_bk}(V - E_{Ca})\]

**Sodium-calcium exchange current (INCX)**

INCX formulation is the same as in the rabbit model with \(v_{NaCa}=3.6\) pA/pF.

**Slowly inactivating delayed rectifier potassium current (IK,slow)**

\[I_{K,slow} = I_{K,slow1} + I_{K,slow2}\]

\[I_{K,slow1} = g_{K,slow1} \cdot x_{K,slow1} \cdot y_{K,slow1}(V - E_K)\]

\[I_{K,slow2} = g_{K,slow2} \cdot x_{K,slow2} \cdot y_{K,slow2}(V - E_K)\]

\[x_{K,slow} = \frac{1}{1 + e^{-\frac{V+15}{14}}},\]

\[\tau_{x_{K,slow}} = 0.95 + 0.05 \cdot e^{-0.08V}\]

\[y_{K,slow} = \frac{1}{1 + e^{-\frac{V+48}{6.2}}},\]

\[\tau_{y_{K,slow1}} = 400 + 900 e^{-\left(\frac{V+55}{16}\right)^2} - \frac{250}{1 + e^{-\frac{V+60}{8}}},\]

\[\tau_{y_{K,slow2}} = 400 + 900 e^{-\left(\frac{V+55}{16}\right)^2} - \frac{550}{1 + e^{-\frac{V+60}{8}}},\]

\[
\frac{dx_{K,slow}}{dt} = \frac{x_{K,slow} - x_{K,slow}}{\tau_{x_{K,slow}}},
\]

\[
\frac{dy_{K,slow1}}{dt} = \frac{y_{K,slow} - y_{K,slow1}}{\tau_{y_{K,slow1}}},
\]

\[
\frac{dy_{K,slow2}}{dt} = \frac{y_{K,slow} - y_{K,slow2}}{\tau_{y_{K,slow2}}},
\]

where \(g_{K,slow1}=0.0352\) nS/pF and \(g_{K,slow2}=0.028\) nS/pF.
Non-inactivating steady-state current ($I_{ss}$)

$$I_{ss} = g_{ss}x_{ss}(V - E_K),$$

where

$$x_{ssss} = x_{klowss}$$

$$\tau_{xss} = 14 + 70e^{-\left(\frac{V+43}{30}\right)^2}$$

$$\frac{dx_{ss}}{dt} = \frac{x_{ssss} - x_{ss}}{\tau_{xss}}$$

D. Numerical methods

The differential equations for voltage and Ca concentrations of different compartments were numerically solved using an Euler method with a time step of 0.1 ms. The gating variables were integrated using the method by Rush and Larsen (14). The LCCs were simulated by a standard Monte Carlo method using the Markov model shown in FIGURE. The RyRs were simulated using an optimized method developed by Restrepo et al (12), which is equivalent to the binomial τ-leaping method (15). For all the simulations in this study, we pre-paced the cell model 50 beats to reach the steady state. All computer programs were coded in CUDA C, and simulations were carried out on a Graphical Processing Unit workstation with 2 intel Xeon E5-26640 processors and 4 Nvidia Tesla K20 GPUs. To simulate 1 s of electrical and Ca cycling activity, it takes $\approx 10$ s of computational time.
Supplemental Figures
(Complex EAD behaviors observed in experiments from literature)

Fig S1. Complex EAD behaviors from experiments. A. EADs with growing amplitude. From Liu et al (3). B. EADs with decreasing amplitude. From Xie et al (9).
Fig. S2. Complex EAD behaviors from experiments. A. EADs with decreasing and then increasing amplitude and long phase-2 plateau. From Ortí et al. (1). B. Long phase-2 plateau followed by EADs with growing amplitude. From Puisieux et al. (8). C. EAD bursts. Upper trace: optical voltage trace. Lower trace: Frequency of the EAD burst. From Change et al. (10).
**Fig. S3. Complex EAD behaviors from experiments.** A. A recording from a dog Purkinje fiber showing complex EAD patterns and irregular beat-to-beat change (Courtesy of Robert Gilmour). B. Alternating EAD pattern following a long phase-2 plateau. During the phase-2 plateau, intracellular Ca is high without oscillation. From Spencer et al. (4).
Supplemental Results

**Fig. S4. Model properties.** A. AP, \([\text{Ca}]_{\text{SR}}, [\text{Ca}]_{\text{i}}\), and a line scan of \([\text{Ca}]_{\text{i}}\) for the normal control model after steady state periodic pacing at PCL=2 s. B. \([\text{Ca}]_{\text{i}}, [\text{Ca}]_{\text{SR}}, I_{\text{NaK}}\) and \(I_{\text{NCX}}\) for \([\text{Na}]_{\text{i}}=7\) mM (black) and 10 mM (red) obtained under voltage clamp condition using the AP in A and PCL=2 s. C. Upper panel: Steady-state activation (\(\text{act}_{\text{ss}}\)) and inactivation (\(\text{inact}_{\text{ss}}\)) curves of \(I_{\text{Ca,L}}\). The red curve is the \(\text{act}_{\text{ss}}\) with 5 mV left-shift. Middle and lower panels: \(I_{\text{Ca,L}}\) and \([\text{Ca}]_{\text{i}}\) before and after a 5 mV left-shift of \(I_{\text{Ca,L}}\) steady-state activation curve under the same voltage clamp condition as in B. D. AP, \(I_{\text{Ca,L}}\), and \([\text{Ca}]_{\text{i}}\) under free running condition (paced with PCL=2 s). The parameters are the same as in C. Note that under AP clamp conditions, 5 mV shift in \(\text{act}_{\text{ss}}\) has very small effect on \(I_{\text{Ca,L}}\) and \([\text{Ca}]_{\text{i}}\) but under free running condition, EADs occur and the changes of \(I_{\text{Ca,L}}\) and \([\text{Ca}]_{\text{i}}\) are large. The normal control model was used in panels A and B, and the L-type Ca channel open probability was increased (the same as in Fig.1 in the main text) in panels C and D.

**Fig.S5.** The entire optical recording from which Fig.6B in the main text (from 40 s to around 72 s) was taken.
Fig.S6. Complex EAD-DAD dynamics in a mouse ventricular model. A. Time traces of voltage, whole cell averaged $[\text{Ca}]_i$ and the corresponding line scan for control conditions. PCL = 1 sec, and $[\text{Ca}]_s = 1 \text{ mM}$. B. pacing-induced EADs. $[\text{Ca}]_s = 2.7 \text{ mM}$ and the maximum conductance of $I_{\text{Na,L}} = 2.7 \text{ pA/pF}$. C. Spontaneous APs (indicated by asterisk) and EADs due to spontaneous Ca release. $[\text{Ca}]_s = 2.7 \text{ mM}$, the maximum conductance of $I_{\text{Na,L}} = 1.2 \text{ pA/pF}$, with doubled the RyR leakiness, increased SERCA activity increased by 50%, and L-type Ca current by 50%. The mouse ventricular cell model were based on the model by Morrotti et al (5), as described in detail in the online supplemental text. The changes in B and C from control were under the assumption that Ca overload causes CaMKII activation which causes the corresponding changes to late $I_{\text{Na,L}}$, RyR, and SERCA.
Fig S7. Interactions of voltage oscillations and Ca oscillations during repolarization failure. Shown are voltage, $[\text{Ca}]_i$ and $[\text{Ca}]_{\text{jsr}}$ versus time, and line scan of $[\text{Ca}]_i$. The arrow in each case indicates the time when voltage clamp started. The parameters are the same as in Fig.1A in the main text but Ca overload ($[\text{Ca}]_o=5$ mM) with $[\text{Na}]_i=10$ mM and 7 mV left-shift in act of $I_{\text{Ca,L}}$. A. The free-running voltage case in which repolarization failure occurs. B. Constant voltage clamp with normal LCCs present in CRUs. C. Same as B, but with a constant uniformly distributed $I_{\text{Ca,L}}$. D. Sinusoidal voltage clamp ($V(t)=A\sin2\pi ft$ with $A=20$ mV and $f=4$ Hz) with normal LCCs present in CRUs. E. Same as D but with a constant uniformly distributed $I_{\text{Ca,L}}$. F. Voltage and $[\text{Ca}]_i$ versus time for a sinusoidal voltage clamp with normal LCCs present in CRUs, at different driving frequencies. Upper two panels: $f=2$ Hz. Lower two panels: $f=1$ Hz.
Interactions between voltage and Ca cycling during the long AP plateau (Detailed description of Figs. S7 and S8)

To explain the observation shown in the experiments in Fig. 6 and the corresponding simulation in Fig. 7 in the main text, in which no Ca oscillations (or very small fluctuations) in the long plateau phase but oscillations occur during the EAD phase, we carried out simulations (Figs. S7 and S8) by clamping the voltage during the AP plateau, so that we could observe the intrinsic underlying Ca cycling dynamics. We chose a parameter setting in which repolarization failure occurred such that voltage during the plateau remained around ~−5 mV, as illustrated in Fig. S7A. When no voltage clamp was imposed, voltage and whole-cell Ca exhibited small but irregular oscillations, and [Ca] remained high. The line scan of Ca shows a random spatiotemporal pattern. We hypothesized that the random spatiotemporal pattern of Ca release under these conditions was likely to be related to the random openings of LCCs producing a random spatial pattern of Ca release sites during the plateau. To investigate how random opening of LCCs affects Ca oscillations, we carried out the following voltage clamp simulations. After starting each simulation from identical initial conditions, we imposed at a certain time point (arrow) either a voltage clamp to a constant voltage (Figs. S7B-C) or a voltage oscillation around the mean voltage (Figs. S7D-F).

When the voltage was held constant to promote random unsynchronized LCC openings, the whole-cell Ca and line scan (Fig. S7B) were very similar to the free-running case of repolarization failure (Fig. S7A). However, if LCCs were removed from the CRUs such that they could no longer trigger Ca sparks directly, and
were replaced by a uniformly-distributed constant Ca flux of the same magnitude as the LCC current so as to maintain the same cellular Ca level, a clear well-organized oscillation pattern immediately developed, as seen in the whole-cell Ca and the line scan (Fig. S7C). Even if the LCCs were removed without replacing them with a uniformly distributed constant Ca flux, Ca oscillations still developed immediately, but subsequently decreased in amplitude as the cellular Ca level declined due to the reduction in Ca entry (Fig. S8). Alternatively, if the voltage was held at a higher voltage to completely inactivate the LCCs, similar Ca oscillations occurred (Fig. S8).

If, instead of holding voltage constant during the plateau, a sinusoidal voltage clamp was imposed, the random spatiotemporal pattern of Ca sparks was replaced by a Ca oscillation pattern at the same frequency as the voltage oscillation (Fig. S7D), but with an alternating pattern. If the LCCs were removed and substituted by an equivalent constant Ca flux (Fig. S7E), however, the Ca oscillations did not alternate and exhibited an oscillation pattern virtually identical to that for the constant voltage case (Fig. S7C). However, now the periodic Ca oscillations were decoupled from the voltage oscillations, such that the frequencies of Ca and voltage oscillations were completely different. Fig. S6F shows [Ca], for different oscillating frequencies of voltage with LCC intact (as in Fig. S7D). As the voltage oscillation frequency decreased, Ca alternans disappeared and [Ca] developed a regular pattern (Fig. S7F, upper trace). However, if the driving frequency was decreased further, an alternating Ca oscillation pattern occurred again (Fig. S7F, lower trace).

These simulations demonstrate the following. When voltage is constant or nearly constant (Fig. S7B), the openings of the LCCs are random and uniform in space and time, resulting in random firings of CRUs in space and time. Therefore, at any time point, a spatially random portion of CRUs have yet to recover from their preceding firing. This random distribution of refractory CRUs reduces the likelihood that excitable CRUs activated by random openings of their LCCs or RyRs will be able to recruit neighboring CRUs and synchronize their refractory periods as required to generate an organized Ca oscillation. This is similar to the mechanism described in our previous study (2) showing that Ca waves and oscillations are emergent properties of the CRU network which strongly depend on CRU recruitment. On the other hand, when the LCCs are removed and replaced by an equivalent Ca flux (Fig. S7C), the number of randomly-firing CRUs causing dispersion of CRU refractoriness are reduced. This allows more effective recruitment to occur, which synchronizes the CRUs for organized Ca oscillations.

For the cases in which voltage oscillates (Figs. S7D-F), fewer LCCs are available for opening at high voltages but more are available at low voltages, and thus the voltage oscillation causes synchronous opening and closing of LCCs, resulting in synchronous firing of the CRUs, accounting for the organized Ca release patterns shown in Fig. S7D. However, if the driving frequency is fast so that the CRUs have not completely recovered from their previous firing, Ca alternans results, similar to the refractoriness mechanism of Ca alternans caused by rapid pacing with a free-running AP (16). When the driving frequency became slower so that CRUs had time to fully recover between oscillations, Ca alternans disappeared (Fig. S7F, upper traces). At even slower driving frequencies (Fig. S7F, lower traces), spontaneous synchronous CRU firings occurred between two pacing beats, resulting in a spontaneous beat followed by a voltage-driven beat, which is another type of Ca alternans.
References


Multiscale determinants of DAD emergence in cardiac tissue
For arrhythmias like VF to occur, an arrhythmia trigger must encounter a tissue substrate vulnerable to the initiation and maintenance of sustained arrhythmias. Though arrhythmia triggers and substrate vulnerability are classically thought to be influenced by separate processes, we have demonstrated in our previous studies that EADs produced at the cellular scale can enhance the susceptibility to reentrant arrhythmias at the tissue scale through mechanisms of dynamical chaos\textsuperscript{32}. Whether DADs, which form due to stochastic processes that produce spontaneous SR Ca release events at the subcellular scale, can also influence the susceptibility to arrhythmias at the tissue scale is not as well understood. Therefore, in this current study, we aim to elucidate how multiple Ca-mediated processes at the subcellular, cellular, and tissue scales are able to generate DADs of sufficient amplitude at the tissue scale to overcome electrotonic source-sink effects and produce triggered action potentials. In order to accomplish this, we combine both experimental and computational modeling approaches to investigate quantitatively how multiscale factors, namely the number of simultaneous Ca waves at the subcellular scale, SR Ca load at the cellular scale, and latency period at the tissue scale, contribute to DAD amplitude in tissue. Here, we use patch clamp electrophysiology and concurrent Ca imaging techniques to measure properties of Ca transients and their corresponding DADs from isolated rabbit ventricular myocytes. We then implement these properties into simplified 25x25 myocyte tissue models to determine the effect of each factor on DAD amplitude. Our findings suggest that the latency period at the tissue scale has the greatest influence on DAD amplitude more so than do the factors at the subcellular and cellular scales.
Figure 5.0. Calcium-mediated DADs and arrhythmias.

A. and B. Spontaneous SR Ca release in myocytes can produce DADs, which, if sufficient in amplitude and electrotonic strength to overcome the sink-effect of neighboring myocytes in neighboring tissue, can form arrhythmia triggers at the tissue scale.

C. The synchrony of Ca release among myocytes in tissue is a major factor influencing DAD amplitude in tissue. Decreased latency periods to Ca wave initiations following rapid pacing trains that increase SR Ca load are associated with greater synchrony of Ca release. Latency durations are determined by the time for SR refilling, CRU recovery from refractoriness, and an idle period, which is likely determined by the time for Ca spark clusters of sufficient size to form to initiate Ca waves.
From Ca Waves to Triggered Activity in Heart:
Scaling from the Subcellular to the Tissue Level

5.1 ABSTRACT

Rationale: Spontaneous calcium (Ca) waves in cardiac myocytes underlie delayed afterdepolarizations (DADs) which can trigger cardiac arrhythmias. How these subcellular/cellular events overcome source-sink factors in cardiac tissue to generate DADs of sufficient amplitude to trigger action potentials (APs) is not fully understood.

Objective: To evaluate quantitatively how factors at the subcellular scale (number of Ca wave initiation sites), cellular scale (sarcoplasmic reticulum (SR) Ca load) and tissue scale (synchrony of Ca release in populations of myocytes) determine DAD features in cardiac tissue.

Methods and Results: Isolated patch-clamped rabbit ventricular myocytes loaded with Fluo-4 to image intracellular Ca were rapidly paced during exposure to elevated extracellular Ca (2.7 mmol/L) and isoproterenol (0.25 µmol/L) to induce diastolic Ca waves and subthreshold DADs. As the number of paced beats increased from 1 to 5, SR Ca content (assessed with caffeine pulses) increased, the number of Ca wave initiation sites increased, integrated Ca transients and DADs became larger and narrower, and the latency period to the onset of Ca waves shortened with reduced variance. In silico analysis using a computer model of ventricular tissue incorporating these experimental measurements revealed that whereas all of these factors promoted larger DADs with higher probability of generating triggered activity, the latency period variance had the greatest influence.

Conclusions: At the tissue level, increased intracellular Ca promotes DAD-mediated triggered activity predominantly by increasing the synchrony (decreasing latency variance) of Ca waves.

Key Words: Calcium wave, delayed afterdepolarization, arrhythmia
**Nonstandard Abbreviations and Acronyms:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaT</td>
<td>Calcium transient</td>
</tr>
<tr>
<td>CPVT</td>
<td>Catecholaminergic polymorphic ventricular tachycardia</td>
</tr>
<tr>
<td>CRU</td>
<td>Calcium release unit</td>
</tr>
<tr>
<td>DAD</td>
<td>Delayed afterdepolarization</td>
</tr>
<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
</tr>
<tr>
<td>LTCC</td>
<td>L-type calcium channel</td>
</tr>
<tr>
<td>PVC</td>
<td>Premature ventricular complex</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SCD</td>
<td>Sudden cardiac death</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TA</td>
<td>Triggered activity</td>
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5.2 INTRODUCTION

Delayed afterdepolarizations (DADs) in cardiac tissue are transient depolarizations of the diastolic membrane voltage which can produce both triggers and a vulnerable substrate for focal and reentrant arrhythmias\(^1\). They constitute a major arrhythmogenic mechanism in the settings of heart failure, catecholaminergic polymorphic ventricular tachycardia (CPVT), digitalis toxicity, and other conditions in which intracellular calcium (Ca) cycling becomes unstable\(^2\). DADs are intimately linked to intracellular Ca waves originating from the subcellular Ca release unit (CRU) network in cardiac myocytes, in which the CRUs consist of L-type Ca channels (LTCCs) in the t-tubule membrane apposed to ryanodine receptors (RyRs) at the sarcoplasmic reticulum (SR) membrane. Under conditions of Ca overload, groups of CRUs which spontaneously release SR Ca at one or more subcellular locations can initiate Ca waves that propagate regeneratively throughout the CRU network of the myocyte. The resulting increase in cytoplasmic free Ca stimulates Ca-sensitive inward currents, primarily the Na-Ca exchange current \(I_{\text{NCX}}\), which causes a transient membrane depolarization. If the DAD is large enough to reach the activation threshold for an action potential (AP), one or a series of APs (triggered activity) can be elicited to initiate focal and/or reentrant arrhythmias\(^3\).

The time course and amplitude of a DAD in an isolated myocyte is shaped by two components: 1) the time course and amplitude of the integrated whole-cell Ca transient caused by the underlying Ca wave(s) and 2) the sensitivity of the resting voltage to changes in intracellular Ca, the so-called diastolic Ca-voltage coupling gain\(^4\). The latter component is determined by the ratio of inward current densities of \(I_{\text{NCX}}\) and other Ca-sensitive currents promoting depolarization to outward current densities opposing depolarization, primarily the inward rectifier K channel, \(I_{\text{K1}}\), in ventricular muscle and the His-Purkinje system. The former component, however, is complex. At the subcellular scale, the location and number of initiation sites of Ca waves is a key determinant of the amplitude, rate-of-rise, and duration of the whole-
cell Ca transient. For example, a Ca wave originating from the center of a myocyte will release SR Ca in half the time of a Ca wave originating from one end of the cell (assuming the same propagation speed), producing an integrated whole-cell Ca transient with approximately twice the amplitude, rate-of-rise, and half the width. In general, as the number of initiation sites increases, the whole-cell Ca transient becomes larger in amplitude and narrower in duration. At the cellular scale, increases in SR Ca content augment the total amount of Ca released and the wave propagation speed, also causing a larger and narrower integrated whole-cell Ca transient. Whereas these factors are clearly major determinants of whether a DAD reaches sufficient amplitude to trigger an AP in an isolated myocyte, at the tissue scale, source-sink (loading) factors also come into play. Since each ventricular myocyte is coupled through gap junctions to an average of 11 other myocytes, a single myocyte exhibiting a Ca wave will be voltage-clamped by neighboring quiescent myocytes, attenuating the DAD amplitude by more than an order of magnitude. Only when a large number of myocytes in a region of tissue all develop Ca waves quasi-synchronously within an overlapping time window can the source-sink mismatch be overcome to generate a DAD of appreciable magnitude in the tissue. Thus, after rapid pacing in cardiac tissue, the timing of the onset of Ca waves (latency period) in adjacent myocytes plays a key role. If the latency period has a large variance, the Ca waves in the individual myocytes may not summate effectively to overcome the source-sink mismatch and generate a DAD of appreciable amplitude.

The goal of this study was to evaluate quantitatively, using a combined experimental and computational approach, the relative importance of these subcellular, cellular, and tissue scale factors as determinants of DAD amplitude and time course in cardiac tissue. Accordingly, we induced Ca waves and DADs by pacing isolated patch-clamped rabbit ventricular myocytes and characterized the number of Ca wave initiation sites, SR Ca content, and latency to onset of Ca waves as the pacing train duration increased. We then incorporated these experimental data
into simulated cardiac tissue to evaluate their relative importance with respect to DAD amplitude, morphology, and likelihood of triggering an AP in tissue.

5.3 METHODS

5.3.1 Ventricular myocyte isolation

Young adult (3-4 months age) New Zealand white male rabbits (1.7–2.0 kg) were euthanized by an intravenous injection of heparin sulfate (1000 U) and sodium pentobarbital (100 mg/kg). Hearts were quickly excised by thoracotomy and retrogradely perfused at 37 °C in Langendorff fashion with Ca-free Tyrode’s solution for 5-7 minutes followed by enzyme digestion perfusion with Tyrode’s solution containing 0.05 mg/mL Liberase TH (Roche) for 20-30 minutes at 25 mL/min. The Tyrode’s solution contained the following (mmol/L): 136 NaCl, 5.4 KCl, 0.33 NaH₂PO₄, 1.0 MgCl₂, 10 HEPES, and 10 glucose; pH 7.4 (KOH). Myocytes were separated from digested ventricles by gentle mechanical dissociation in 0.2 mM Ca Tyrode’s solution. Ca concentration was gradually increased to 1.8 mmol/L over 30 min. Myocytes were used within 6-8 hours. All procedures complied with UCLA Animal Research Committee policies.

5.3.2 Patch clamp recordings

Standard whole-cell patch-clamp methods were used to measure voltage and current in the current clamp or voltage clamp mode, respectively. Borosilicate glass electrodes (tip resistance 1.4-2.2 MQ) were filled with internal solution containing (in mmol/L) 110 K-Aspartate, 30 KCl, 5 NaCl, 10 HEPES, 5 MgATP, 5 creatine phosphate, 1 KH₂PO₄, and 0 EGTA; pH 7.2 (KOH). Fluo-4 pentapotassium salt (10 µmol/L) (Life Technologies) and the reducing agent glutathione (GSH) (10 mmol/L) were added to the internal solution to visualize Ca fluorescence.
activity while minimizing the effects of phototoxic damage. APs were elicited with square current pulses of 2 ms duration and twice threshold amplitude. DADs were evoked following rapid pacing trains of varying durations in the presence of Tyrode’s solution with elevated Ca (2.7 mmol/L) and isoproterenol (ISO) (0.25 µmol/L). Data were acquired with an Axopatch 200A patch-clamp amplifier and Digidata 1200 acquisition board driven by pCLAMP 9.0 software (Axon Instruments, Inc.). Corrections were made for liquid junction potentials. Signals were filtered at 1 kHz. All experiments were carried out at 37 °C. All chemicals were purchased from Sigma unless indicated otherwise.

5.3.3 Calcium imaging

Ca fluorescence in patch-clamped myocytes was imaged using an inverted Nikon Diaphot microscope (60X objective, Olympus) equipped with a charge-coupled device (CCD)-based Photometrics Cascade 128+ camera (~100 frames/s, 128x128 pixels) operating under Imaging Workbench software (version 6.0, INDEC BioSystems). Pseudo line scan images were generated from the acquired video data using ImageJ software. All Ca fluorescence data were corrected for bleaching and then normalized to the peak amplitude of the Ca transient of the first AP in the AP train.

5.3.4 Rapid caffeine solution delivery

To measure SR Ca content, patch-clamped myocytes were rapidly superfused with Tyrode’s solution containing 10 mmol/L caffeine, 2.7 mmol/L Ca, and 0.25 µmol/L ISO using a rapid solution exchange device positioned near the myocyte under the control of Axopatch software.
5.3.5 Mathematical model of cardiac tissue and simulations

Tissue simulations in 1D (25 myocytes), 2D (25x25 myocytes), and 3D (25x25x25 myocytes) were carried out using the rabbit ventricular AP cell model previously described in Mahajan et al. The partial differential equation governing voltage for the 2D tissue is

\[
\frac{\partial v}{\partial t} = -I_{ion}/C_m + D\left(\frac{\partial^2 v}{\partial x^2} + \frac{\partial^2 v}{\partial y^2}\right) \tag{1}
\]

where \(v\) is the membrane voltage, \(C_m\) is the membrane capacitance (1 \(\mu\)F/cm\(^2\)), and \(D\) is the gap junction diffusion constant (0.000557 cm\(^2\)/ms). For numerical calculations, an operator splitting and time adaptive algorithm was used with the time step (\(\Delta t\)) varying from 0.01 ms to 0.1 ms.

DADs were generated by commanding an intracellular Ca transient modeled as a Gaussian-shaped function in time as follows

\[
[Ca]_i = A \cdot e^{-(t-t_0)^2/2\sigma^2} \tag{2}
\]

where \(A\) is the maximum amplitude of the transient, \(t_0\) sets the latency of the DAD, and \(\sigma\) controls the width of the Ca transient. To convert from measured full width at half maximum (FWHM) values to \(\sigma\) we used the standard equation for a Gaussian \(\sigma = \text{FWHM} / (2\sqrt{2\ln 2})\). Once an AP upstroke was detected, the Ca clamp waveform was turned off and Ca was allowed to run freely in the model. The amplitude, duration, and latency period of the Ca transient waveform were varied to simulate the experimentally-measured values. Assignment of latency periods to different myocytes in tissue simulations was accomplished by a bootstrap method, randomly selecting latency periods from the distribution of actual experimental data points corresponding to the low and high Ca load cases summarized in Table 5.1A.

5.3.6 Data Analysis and Statistics

Data in this study are presented as medians and 95% confidence intervals (CIs). The conventional percentile bootstrap-resampling approach with 10,000 replications was used for
estimating 95% CIs. All analyses were performed by subroutines for bootstrapping developed in the Python programming language, using the Numpy and Scipy packages, based on our previously published code\textsuperscript{16-18}.

5.4 RESULTS

5.4.1 Post-pacing Ca waves and DADs in isolated ventricular myocytes

Isolated rabbit ventricular myocytes were exposed to modestly elevated extracellular Ca (Ca\textsubscript{o}) (2.7 mmol/L) and ISO (0.25 µmol/L) and paced for 1 to 5 beats at a cycle length of 400 ms. After each pacing train, the myocyte was left unstimulated for 5 s during which intracellular Ca was imaged and membrane voltage recorded to detect Ca waves and DADs respectively, as illustrated in Fig. 5.1. In this myocyte, a single pacing stimulus (left-most column) elicited an AP and Ca transient, followed by a barely perceptible DAD (top row) associated with a small Ca transient after a long latency period (2\textsuperscript{nd} row) caused by a Ca wave arising from a site of origin near the middle of the cell (4\textsuperscript{th} row, red dot) which propagated only partially through the cell (3\textsuperscript{rd} row). As the number of paced beats in the train was increased from 2 to 5 (subsequent columns), DADs due to Ca waves arising from multiple sites occurred following the last paced beat (indicated by the vertical red line) in each train. With longer pacing trains, Ca waves began to appear during the pacing train (not shown), complicating the analysis.

The experimental data on DAD and Ca transient characteristics including amplitude, duration (full-width-at-half-maximum, FWHM), and rate of rise for 7 myocytes subjected to the same pacing train protocol in Fig. 5.1 are summarized in Fig. 5.2. Since the number of paced beats required to elicit a DAD varied from myocyte to myocyte and even from trial to trial due to the stochastic nature of spontaneous SR Ca release, the experimental DAD and Ca transient characteristics were compared relative to the minimum number of paced beats required to elicit the first post-pacing DAD (ranging from 1, as in Fig. 5.1, to 4). Hence, the horizontal axis label
“+0” refers to the minimum number of paced beats required to elicit a DAD, “+1” refers to the
DAD elicited by one additional paced beat over the minimum number, “+2” refers to the DAD
elicited by two additional paced beats over the minimum, and so forth. The results show that as
the number of additional paced beats increased and progressively loaded SR Ca stores (see
later), DAD amplitude increased, its duration shortened, and its rate of rise increased (Fig.
5.2B), roughly in parallel with the changes in integrated whole-cell Ca wave transients (Fig.
5.2A).

In 2 of the 7 myocytes, the first DAD elicited by the minimum number of paced beats (the
“+0” case in Fig. 5.2) resulted from multiple Ca wave initiation sites, instead of a single site as in
Fig. 5.1. Therefore, we also analyzed the DAD and Ca transient characteristics according to the
number of Ca wave initiation sites underlying the Ca transient and DAD. As shown in Fig. 5.3,
as the number of Ca wave initiation sites increased, the resulting Ca transient (Fig. 5.3A) and
DAD amplitude (Fig. 5.3B) increased, the duration shortened, and the rate of rise increased.

5.4.2 Post-pacing Ca transient and DAD latency period distributions

The latency period, defined as the time interval from the last pacing stimulus to the onset
of the first post-pacing Ca wave or DAD, also shortened as the number of paced beats in the
train increased from +0 to +4, as shown in Fig. 5.4A. The variance also decreased dramatically.
When the data was analyzed according to the number of Ca wave initiation sites (analogous to
Fig. 5.3), both the latency period and its variance decreased dramatically as the number of Ca
wave initiation sites increased (Fig. 5.4B).

5.4.3 Post-pacing intracellular Ca load

The changes in Ca transient and DAD amplitude, duration, rate of rise, and latency
period distributions observed in Figs. 5.2-5.4 are presumably related to progressive intracellular
Ca loading as the number of paced beats in the train increased\textsuperscript{11, 12}. For example, it is apparent from the traces in Fig. 5.1B that diastolic Ca fluorescence did not return fully to the baseline between paced beats, consistent with the total intracellular Ca increasing with the number of paced beats. To estimate quantitatively the increase in SR Ca load as the number of paced beats increased, 5 voltage-clamped myocytes were paced with an AP clamp waveform. After the last AP waveform in the train (ranging from 1 to 5 beats), a caffeine pulse (10 mmol/L for 1 s) was rapidly applied to release SR Ca, and the resulting inward current due to $I_{\text{NCX}}$\textsuperscript{3} was recorded simultaneously with the caffeine-induced Ca transient (Fig. 5.5A). The inward current (Fig. 5.5B) was integrated from the onset of caffeine exposure until return to the diastolic baseline (or a maximum duration of 3 s) to estimate the charge movement related to Ca extrusion by Na-Ca exchange. Fig. 5.5C shows that the intracellular Ca released by caffeine increased progressively with the number of paced beats in the train, from 0.00790 [0.00397, 0.0119] fmol/pF following a single paced beat to 0.01540 [0.01390, 0.01640] fmol/pF after 5 paced beats, a 1.8-fold increase.

5.4.4 Incorporation of experimental Ca wave and DAD characteristics into simulated 2D cardiac tissue

The experimental findings above demonstrate that as the intracellular Ca load increased with longer pacing trains (Fig. 5.5), post-pacing Ca transients and DADs became greater in amplitude and shorter in duration with a faster rate of rise (Fig. 5.1-5.3) and also occurred earlier with a narrower latency period distribution (Fig. 5.4). To estimate quantitatively the degree to which each of these factors influences DAD characteristics in tissue in which source-sink effects come into play\textsuperscript{6}, we simulated a 2D cardiac tissue (25x25 myocytes) using a rabbit ventricular AP and Ca cycling model\textsuperscript{15}. To generate DADs, the model was equilibrated to pacing at a cycle length of 400 ms, and one of two idealized intracellular Ca transient waveforms was
commanded for each cell at a pre-specified latency period to stimulate \( I_{\text{NCX}} \) and evoke a DAD. For this purpose, the experimental data were categorized into either a low or a high intracellular Ca load case (Table 5.1). For the low Ca load case, Ca transients were modeled to have a long duration with FWHM of 291 ms corresponding to the pooled median duration for 1-2 sites of Ca wave initiation (Fig. 5.3A), a “low” amplitude corresponding to the relative amount of caffeine-induced SR Ca release following 1 paced beat (Fig. 5.5), and latencies bootstrapped from the pooled data for 1-2 sites of Ca wave initiation which exhibits a “broad” distribution (Fig. 5.4B). For the high Ca load case, Ca transients were modeled to have a “short” duration of 137 ms corresponding to the pooled median duration for 3-7 sites of Ca wave initiation (Fig. 5.3A), “high” amplitude potentiated by a 1.8-fold greater SR Ca content than the low Ca load case (based on the relative increase in SR Ca load after 5 paced beats in Fig. 5.5), and latencies bootstrapped from the pooled data for 3-7 sites of Ca wave initiation which exhibits a “narrow” distribution (Fig. 5.4B). With these settings, the low and high intracellular Ca load cases produced DAD amplitudes of 1.4 and 5.0 mV, respectively, when the diastolic Ca-voltage coupling gain of the AP model was adjusted to mimic the experimentally measured values of median DAD amplitudes for 1-2 sites and 3-7 sites, respectively (Fig. 5.3B). The median and 95% confidence intervals for SR Ca loads and latency periods for the low and high Ca load conditions are listed in Table 5.1A.

In order to assess how the features of the Ca transient waveforms and DADs in uncoupled myocytes scale to the tissue level in which source-sink relationships are important\(^6\), we next implemented the idealized Ca transient waveforms corresponding to the high and low Ca load cases into a virtual tissue of 25x25 myocytes. For convenience, Ca-voltage coupling gain was adjusted to produce sizeable, but still subthreshold, DADs with peak amplitudes of 6.6 and 16.0 mV, respectively, for the low and high Ca load cases. When the myocytes in the simulated tissue were uncoupled, commanding a Ca transient waveform corresponding to the low Ca load case produced 625 identical DADs of long duration and low amplitude smeared
over a wide temporal range corresponding to randomly selected latencies from the broad
distribution in Table 5.1A (Fig. 5.6A, upper left traces). In comparison, when the Ca transient
waveform corresponded to the high Ca load case, the 625 identical DADs had a shorter
duration and higher amplitude and were more tightly clustered in time corresponding to the
narrow latency distribution (Fig. 5.6A, lower left traces). When cells in the tissue were then
coupled diffusively so that membrane voltage could diffuse, the DAD duration widened, and its
amplitude was attenuated. For the low Ca load case, DAD amplitudes throughout the tissue
decreased by an average of 67% from 6.6 to 2.2 mV and the FWHM increased by 20% to 845
ms (Fig. 5.6A, upper right traces). For the high Ca load case, the DAD amplitudes decreased by
53% from 16.0 to 7.0 mV and the FWHM increased by 15% to 157 ms (Fig. 5.6A, lower left
traces). The greater attenuation of DAD amplitude and rate of rise in the low Ca load case
occurred because the broad latency period distribution caused significant desynchronization of
the Ca waves between myocytes in the tissue, attenuating the peak and smearing the duration
to a greater extent than the high Ca load case with its narrower latency distribution.

To investigate how the low and high Ca load cases affect the ability of DADs to trigger
an AP in tissue, the tissue simulations were repeated 100 times with a different randomly-
selected spatial distribution of latency periods in each trial. The diastolic Ca-voltage coupling
gain was varied by altering the maximal conductance of the Na-Ca exchanger in the model to
change the $I_{NCX}/I_{K1}$ ratio at its resting voltage for each set of 100 trials. For convenience, we
assigned a value of 1.0 to the $I_{NCX}/I_{K1}$ ratio at which the idealized Ca transient for the high Ca
load case triggered an AP in at least one of the 100 trials, indicated in Fig. 5.6B by the sharp
vertical rise in the solid green trace corresponding to the high Ca load case. For the low Ca load
case, the Ca-voltage coupling gain had to be increased by a factor of 3.0 to trigger an AP
(dashed red trace in Fig. 5.6B and Table 5.1B). Thus, in the low Ca load case, the combination
of low amplitude, slow rate-of-rise, long duration Ca waves (due to 1-2 sites of Ca wave
initiation and lower SR Ca content) coupled with a wide latency period distribution markedly
suppressed the resulting DAD’s ability to reach the threshold to trigger an AP in tissue, unless the Ca-voltage coupling gain was markedly enhanced.

5.4.5 Determinants of the probability of a DAD triggering an AP in tissue

We next addressed which of the above parameter differences between the low Ca load and high Ca load cases (DAD duration, DAD amplitude/SR content, or latency variance) had the greatest influence on whether the DAD was able to reach the threshold to trigger an AP. For this purpose, we constructed hypothetical Ca transients possessing hybrid combinations of the three factors from the low and high Ca load cases, generating six hypothetical Ca transients with the properties listed in Table 5.1B. For each case, we performed 100 trials, each with a different randomly-selected spatial distribution of latency periods in the 2D tissue for each Ca-voltage coupling gain value. Figure 5.6B shows that among the three factors, changing the latency period distribution from narrow (solid lines) to broad (dashed lines) had the largest effect at increasing the Ca-voltage coupling gain required for the DAD to trigger an AP in tissue, followed by increasing the SR Ca content by 1.8-fold, followed by increasing the number of Ca wave initiation sites from 1-2 to 3 or more.

The influence of the variance of latency periods on the threshold value of Ca-voltage coupling gain required for a DAD to trigger an AP in simulated 2D tissue is illustrated further in Fig. 5.7, which plots the probability (in 100 trials) of a DAD triggering an AP as the Ca-voltage coupling gain was increased. As the distribution of latency periods was decreased from a standard deviation of 350 ms (equivalent to the low Ca load case) to 50 ms (equivalent to the high Ca load case), the Ca-voltage coupling gain required for a DAD to trigger an AP decreased three-fold.
5.4.6 Effects of tissue dimension and the diffusion coefficient

The simulations in Figs. 5.6 and 5.7 represent 2D cardiac tissue with a physiological diffusion coefficient ($D = 0.000557 \text{ cm}^2/\text{ms}$) representing normal gap junction coupling producing a physiological ventricular conduction velocity. Since source-sink relationships are influenced by tissue dimensionality and diffusivity, we next investigated how these factors affected the ability of DADs to trigger an AP in tissue. Accordingly, the results in simulated 2D tissue (25x25 myocytes) were compared to a 1D cable (25 myocytes) and a 3D slab (25x25x25 myocytes), using Ca wave transients/DADs corresponding to the high and low Ca load cases, as well as two hypothetical cases. Fig. 5.8A shows that the Ca-voltage coupling gain at which DADs triggered an AP was significantly lower in the 1D cable, especially when the latency distribution was broad, whereas the differences between 2D and 3D tissue were small.

Next, we compared the effect of decreasing the diffusion coefficient four-fold (equivalent to reducing gap junction conductance) to halve conduction velocity. As shown in Fig. 5.8B, reducing the diffusion coefficient had almost negligible effects on the Ca-voltage gain required for the DAD (using the low Ca load case) to trigger an AP regardless of tissue dimension.

5.5 DISCUSSION

In this study, we used a combined experimental and modeling approach to analyze the subcellular/cellular/tissue scale factors that determine whether a DAD in cardiac tissue reaches sufficient amplitude to trigger an AP. The major factors that we analyzed include: 1) how quickly the SR Ca is released in each myocyte (i.e. the location and number of sites at which Ca waves are initiated) which defines the peak amplitude and duration of the resulting whole-cell Ca transient activating $I_{\text{NCX}}$ and other Ca-sensitive inward currents (the subcellular scale factor); 2) how much SR Ca is released by each myocyte (i.e. the SR Ca load representing the cellular
scale factor); 3) how synchronously Ca waves in adjacent myocytes release their SR Ca (i.e. the latency period distribution) required to overcome the source-sink mismatch (the tissue scale factor); and 4) how sensitive the membrane voltage is to the increase in cytoplasmic free Ca (the diastolic Ca-voltage coupling gain, another cellular factor). Our major finding is that for a given Ca-voltage coupling gain, the most influential factor determining whether a DAD in tissue reaches the threshold to trigger an AP is the latency period variance determining the synchrony of cellular Ca waves in a region of tissue. It has previously been shown in confocal imaging studies in intact rat ventricular muscle that isolated Ca waves in individual myocytes during slow pacing caused no detectable changes in membrane potential due to the source-sink mismatch, and only when the majority of myocytes developed Ca waves synchronously did detectable DADs and triggered activity result\textsuperscript{10,11}. Here we show in simulated tissue that even when 100% of the myocytes in the tissue develop Ca transients after a paced beat, the synchronicity of Ca waves still remains the predominant factor determining whether the DAD reaches sufficient amplitude to trigger an AP. This conclusion agrees with the recent simulation study examining the DAD threshold for triggering an AP as SR load was progressively increased in an anatomic rabbit ventricles model\textsuperscript{8}. However, in this model, SR Ca load, DAD peak amplitude, rate-of-rise, width and latency all changed simultaneously, so that the individual contributions of the subcellular, cellular and tissue factors were not analyzed. In addition, we utilized experimentally measured properties of Ca wave elicited by pacing in rabbit ventricular myocytes to assure that the DAD peak amplitude, rate-of-rise, width, and latency distributions as SR Ca load increased were physiologically realistic rather than purely model-generated. Thus, for physiologically realistic Ca wave properties, our results indicate that the tissue scale source-sink factor is the most critical determinant of DAD amplitude in cardiac tissue, with the amplitude and duration of Ca waves in individual myocytes at the subcellular and cellular scales playing still important but less influential roles. This was true for 1D, 2D and 3D cardiac tissue, whether the diffusion coefficient was normal or reduced.
5.5.1 Biophysical mechanisms underlying Ca waves and DAD features

The mechanisms by which an increased number of Ca wave initiation sites and increased SR Ca load influence DAD amplitude and duration are straightforward. If whole-cell SR Ca content is released over a shorter time period due to the fusion of multiple Ca waves, the peak amplitude will increase and the duration will narrow proportionately, generating a larger and narrower DAD. If more Ca is released over the same time period due to a higher SR content, the DAD peak amplitude will increase proportionately without affecting DAD duration. However, a high SR Ca load also usually accelerates Ca wave speed, in which case the release time will be modestly shortened to cause a narrower DAD. The mechanism underlying the Ca wave latency period distribution, on the other hand, is more controversial. Experimental evidence indicates that both refilling of SR Ca stores and recovery of RyR refractoriness are complete well before the typical onset of a Ca wave, requiring an additional time delay, which has been called the “idle period” prior to the Ca wave initiation. Theoretical analysis supported by experimental data predicts that the idle period arises from criticality in the CRU network\(^{19}\) and reflects the time required for a random cluster of adjacent CRUs to all fire within the same time window, thereby releasing enough Ca locally to initiate a regenerative Ca wave. The time needed to form a critical cluster as well as the critical cluster size becomes smaller as SR Ca load and diastolic cytoplasmic Ca increases, so that the mean time before an appropriately sized cluster forms to initiate a Ca wave (the idle period) becomes shorter with less variance. Thus, as the intracellular Ca load increases, latency period distribution becomes narrower with a shorter median time.

5.5.2 Limitations

We studied the characteristics of Ca waves and DAD induced only by one condition, namely the combination of ISO and modestly elevated extracellular [Ca], and it is possible that
other interventions may give different results. Cytoplasmic dialysis of the Ca-buffering fluorescent dye Fluo-4 as well as other washout of other constituents could also have influenced the Ca wave features. We deliberately studied DADs with amplitudes that were below the threshold for eliciting an AP so that we could characterize the full time evolution of Ca waves and DADs uninterrupted by APs. However, further increasing ISO to 0.5 µM and extracellular [Ca] to 3.6 mM consistently induced post-pacing triggered activity (unpublished observations).

In our simulations, we used DADs that were commanded at the whole cell level which does not account for synchronizing effects of membrane depolarization on Ca wave onset in adjacent cells, effectively making the latency distribution somewhat narrower compared to isolated myocytes. We also did not account for intracellular Ca diffusion from myocyte-to-myocyte through gap junctions, which might have a cooperative effect on the timing of Ca wave initiation in nearby myocytes. Although Ca wave propagation from myocyte-to-myocyte has been observed in myocyte pairs, it is generally a slow process. Whether it would have much influence on latency periods in the range of 400 ms (Fig. 5.4) is unclear, but can be investigated in future simulation studies. Finally, we simulated homogeneous isotropic tissue, in which all the myocytes had identical properties except for the assigned latency period of the Ca transient, whereas real cardiac myocytes and tissue are heterogeneous and anisotropic. Future studies will be required to determine whether these cellular and tissue heterogeneities have important effects.

### 5.5.3 Physiological Implications

Our findings indicate that in cardiac tissue, the latency period distribution of Ca waves is the single most influential factor in determining whether a DAD reaches the threshold to elicit a triggered AP. Thus, the narrowing of the latency period distribution as the intracellular Ca load increases during pacing plays a major role in shaping the rate dependence of DAD amplitude,
coupling interval, and initiation of triggered activity noted in previous experimental studies.\textsuperscript{11, 22}. By desynchronizing the timing of Ca waves in myocytes, the effect of a broad latency distribution is to attenuate the tissue DAD amplitude and prolong its duration in comparison to the DAD generated by the same Ca wave in an uncoupled myocyte. This effect protects normally-coupled 3D tissue from DAD-induced triggered activity.

It is interesting that reducing the diffusion coefficient four-fold, corresponding to a two-fold decrease in conduction velocity, did not significantly affect the Ca-voltage coupling gain required for a DAD to trigger an AP (Fig. 5.8B). Thus, gap junction remodeling that uniformly reduces conduction velocity does not by itself appear to significantly increase the probability of DAD-induced triggered activity in tissue in which 100\% of myocytes exhibit DADs. This is different from the case in which myocytes exhibiting DADs are coupled to myocytes without DADs, as in our previous simulation study.\textsuperscript{6} In this case, reducing gap junction coupling promoted triggered activity by decreasing the current sink effect of the myocytes without DADs.

In contrast to gap junction coupling, reducing tissue dimension from 3D to 1D did significantly lower the Ca-voltage coupling gain required for a DAD to trigger an AP (Fig. 5.8). This may be a factor increasing the susceptibility of fibrotic tissue to DAD-mediated triggered activity, since fibrosis creates myocyte strands acting functionally as quasi-1D cables embedded in 2D or 3D tissue. Similarly, other factors being equal, the Ca-voltage coupling gain required for a DAD to trigger an AP would be lower in Purkinje fibers (also quasi-1D cables) than in 3D myocardium. Once again, it should be noted that in our simulations, we commanded Ca waves/DADs to occur in 100\% of myocytes. When only a portion of the myocytes in the tissue exhibit Ca waves/DADs, the effects of tissue dimension are quantitatively much larger.\textsuperscript{6, 8}

Finally, since real cardiac tissue is heterogeneous both at the cellular and tissue levels, an important consequence of the randomness of latency period distributions is that regions with suprathreshold DADs and subthreshold DADs may coexist in the same tissue. As shown in a recent study,\textsuperscript{23} subthreshold DADs can locally reduce excitability by inactivating the Na current,
leading to conduction block and initiation of reentry. Thus, the same DAD-mediated process can produce both the trigger and vulnerable substrate (due to dispersion of excitability) promoting initiation of reentry and cardiac fibrillation.

5.6 ACKNOWLEDGEMENTS

We thank Guillaume Calmettes, PhD, and Alan Garfinkel, PhD, for their help with the statistical analysis. We also thank Pauline Morand for myocyte preparation.

5.7 FUNDING SOURCES

Supported by NIH/NHLBI grants P01 HL078931 and T32 GM065823 (to C.K.), American Heart Association Western States Affiliate Pre-doctoral Research Fellowship 10 PRE3030052 (to C.K.), UCLA MSTP grant T32 GM008042 (to M.L.) and the Laubisch and Kawata endowments.

5.8 DISCLOSURES

None.
### Table 5.1A. Properties of Ca waves associated with high and low Ca loads

<table>
<thead>
<tr>
<th>Experimental CaT Properties</th>
<th># Ca Waves Initiation Sites</th>
<th>SR Ca load (fmol/pF)</th>
<th>Latency Period (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Ca</td>
<td>1-2</td>
<td>0.0079 [0.0040, 0.0120]</td>
<td>706 [537, 919]</td>
</tr>
<tr>
<td>High Ca</td>
<td>3-7</td>
<td>0.0150 [0.0140, 0.0160]</td>
<td>444 [329, 464]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Implemented CaT Properties</th>
<th>FWHM (ms)</th>
<th>Relative Amplitude</th>
<th>Latency Period (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Ca</td>
<td>291</td>
<td>1</td>
<td>706 [537, 919]</td>
</tr>
<tr>
<td>High Ca</td>
<td>137</td>
<td>1.8</td>
<td>444 [329, 464]</td>
</tr>
</tbody>
</table>

Summary of experimental data in isolated rabbit myocytes (Experimental CaT Properties) from which properties of idealized Ca waves corresponding to low Ca load and high Ca load cases (Implemented CaT Properties) were derived. For the low Ca load case, experimental data corresponds to Ca waves initiated from 1 or 2 sites, with a low SR Ca load and broad latency distribution. For the high Ca load case, experimental data corresponds to Ca waves which were initiated from 3-7 sites, with a 1.8-fold higher SR Ca load and narrow latency distribution. Values shown are the median and 95% CI.

### Table 5.1B. Ca-voltage coupling gain thresholds required to trigger an AP for different Ca transient waveform properties

<table>
<thead>
<tr>
<th>Simulated Combination</th>
<th>Ca Transient Duration</th>
<th>Ca Transient Amplitude (High = 1.8 x Low)</th>
<th>Latency Period Distribution</th>
<th>Ca-V Coupling Gain for Triggered AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothetical CaT #1</td>
<td>Short</td>
<td>Low</td>
<td>Broad</td>
<td>4.0</td>
</tr>
<tr>
<td><strong>Low Ca load CaT</strong></td>
<td>Long</td>
<td>Low</td>
<td>Broad</td>
<td>3.0</td>
</tr>
<tr>
<td>Hypothetical CaT #2</td>
<td>Short</td>
<td>High</td>
<td>Broad</td>
<td>2.1</td>
</tr>
<tr>
<td>Hypothetical CaT #3</td>
<td>Short</td>
<td>Low</td>
<td>Narrow</td>
<td>1.9</td>
</tr>
<tr>
<td>Hypothetical CaT #4</td>
<td>Long</td>
<td>High</td>
<td>Broad</td>
<td>1.5</td>
</tr>
<tr>
<td>Hypothetical CaT #5</td>
<td>Long</td>
<td>Low</td>
<td>Narrow</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>High Ca Load CaT</strong></td>
<td>Short</td>
<td>High</td>
<td>Narrow</td>
<td>1.0</td>
</tr>
<tr>
<td>Hypothetical CaT #6</td>
<td>Long</td>
<td>High</td>
<td>Narrow</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Based on the properties of the high and low Ca load cases (emboldened) summarized in Table 5.1A, six additional hypothetical combinations were generated corresponding to Ca transients (CaT) with short or long duration, high or low amplitude, and narrow or broad latency distribution. The Ca-voltage coupling gain threshold required to trigger an AP in a virtual 2D tissue of 25x25 coupled myocytes (D=0.000557 cm^2/ms) is listed for each case.
Figure 5.1. Induction of Ca waves and DADs by rapid pacing in a representative isolated ventricular myocyte. Ca waves and corresponding DADs elicited from rabbit ventricular myocytes (n=7) exposed to elevated Ca\textsubscript{o} (2.7 mmol/L) and ISO (0.25 µmol/L) following a pacing train from 1 to 5 beats at 400 ms pacing cycle length. **A-B.** Voltage and Ca fluorescence traces illustrating post-pacing DADs and Ca wave transients as the number of paced beats was increased from 1 to 5. Dashed red line indicates the beginning of the last paced beat in a pacing train (red dotted line) from which the latency period to the onset of the first spontaneous Ca wave or DAD was calculated. **C.** Corresponding space-time plots of Ca fluorescence. **D.** Ca wave initiation sites (red dots) corresponding to post-pacing Ca waves.
Figure 5.2. Post-pacing Ca wave transient and DAD properties in 7 isolated myocytes. Ca wave transient (A) and DAD (B) amplitude, duration (FWHM) and rate of rise versus the number of paced beats (see text). Medians and 95% CIs of medians are indicated in blue. Traces at top indicate how measurements were made (red lines).
Figure 5.3. Post-pacing Ca wave transient and DAD properties in 7 isolated myocytes. Ca wave transient (A) and DAD (B) amplitude, duration (FWHM) and rate of rise versus the number of sites initiating the post-pacing Ca wave (see text). Medians and 95% CIs of medians are indicated in blue. Traces at top indicate how measurements were made (red lines).
Figure 5.4. Post-pacing Ca wave transient and DAD latency periods in 7 isolated myocytes. A. Latencies of Ca wave transients (above) and DADs (below) versus the number of paced beats corresponding to Fig. 5.2. B. Same, but as a function of the number of sites initiating the post-pacing Ca wave, corresponding to Fig. 5.3. Medians and 95% CIs of medians are indicated in blue. Trace at top indicates how measurements were made (red lines).
Figure 5.5. SR Ca load versus number of paced beats. A-B. Rabbit ventricular myocytes (n=5) were paced from 1-5 beats with an AP waveform in the voltage clamp mode. Five hundred ms after the last AP, caffeine (10 mmol/L) was rapidly superfused for 1s to release SR Ca. Ca fluorescence (A) and membrane currents (B) were recorded after the last paced AP. C. Ca extruded by NCX during the caffeine pulse, calculated from the integral of \( I_{NCX} \) during the caffeine-induced Ca transient, as a function of the number of paced beats. Medians and 95% CIs of medians are indicated in blue.
Figure 5.6. Simulated DADs and DAD thresholds for triggering an AP in simulated 2D cardiac tissue (25×25 myocytes). A. Superimposed DADs from all 625 myocytes for the low Ca load case (upper traces) and high Ca load case (lower traces) before (left) and after (right) the myocytes in the tissue were diffusively coupled ($D = 0.000557 \, \text{cm}^2/\text{ms}$). B. Maximum DAD amplitude in 2D tissue as a function of the Ca-voltage coupling gain for different combinations of DAD amplitude, SR Ca content, and latency distributions corresponding to the high and low Ca load cases in Table 5.1. Sudden jumps in DAD amplitude indicate the thresholds for triggering an AP.
Figure 5.7. Effect of the latency distribution on the probability of a DAD reaching the threshold to trigger an AP. Using the Ca wave/DAD properties corresponding to the low Ca load case (Table 5.1A) and a Gaussian distribution of latencies in the simulated 2D tissue (25x25 myocytes), the probability of a DAD triggering an AP in 100 trials for each Ca-voltage coupling gain is plotted, as the standard deviation of the latency period distribution is varied from 350 to 50 ms.
Figure 5.8. Effect of tissue dimension and diffusion coefficient on the ability of a DAD to trigger an AP. **A.** The value of the Ca-voltage coupling gain required for a DAD to trigger an AP in 1D (25 myocytes), 2D (25x25 myocytes), and 3D (25x25x25 myocytes) is shown for 4 combinations of DAD properties in Table 5.1: short or long duration, high or low amplitude, and narrow or broad latency distribution. **B.** The value of the Ca-voltage coupling gain required for a DAD to trigger an AP in simulated 2D tissue (25x25 myocytes) for normal and reduced diffusion coefficients of D=0.000557 and 0.000125 cm²/ms, respectively. DAD properties correspond to the low Ca load case in Table 5.1A.
REFERENCES


Discussion
6.1 General comments

Two major hurdles have made the study of cardiac arrhythmias especially challenging. The first is the heart’s electrophysiology, which, due to the many nonlinear interactions inherent in the system, oftentimes produces counterintuitive biological behaviors. The second is the multiscale basis by which properties or functions of one or a group of biological components at one scale of biological organization are able to traverse scales and assert their influence on other scales. In order to overcome these challenges, we applied views and approaches from nonlinear dynamics in both experimental electrophysiology and mathematical modeling contexts to achieve our goal in this body of work, which was to further the understanding of how certain patterns of spontaneous SR Ca release emerge at the subcellular scale and influence DADs and EADs at the cellular and tissue scales. Using this approach, we have uncovered key findings that provide novel insights into both the biophysical and physiological mechanisms of cardiac arrhythmias. In summary, we have demonstrated that 3R interactions within the CRU network give rise to various patterns of subcellular Ca release and, in particular, that the transition from stochastic Ca sparks to organized Ca waves is caused by a universal mechanism of criticality that had not previously been associated with Ca cycling mechanisms in the heart (Chapter 2). We have also shown how the spatiotemporal features of spontaneous SR Ca release events within the CRU network alone are sufficient to give rise to Ca oscillations and pacemaker sites even in the absence of structural heterogeneities within myocytes (Chapter 3). At the cellular scale, we have shown how Ca-voltage coupling can influence the morphology and timing of DADs and even give rise to qualitatively new features of EADs that had not yet been predicted by existing dynamical theories (Chapters 4 & 5). At the tissue scale, we have provided evidence supporting a relatively newer notion that mechanisms that promote the formation of arrhythmia triggers can also directly increase the vulnerability of the tissue.
substrate (Chapters 4 & 5). In the following, we discuss further the relevance and significance of these interdependent influences at each scale.

6.1.1 The dynamic basis of spontaneous SR Ca release in the subcellular CRU network

In our previous studies, we have derived several generalized frameworks, such as the 3R theory, for determining how dynamical interactions among properties of the subcellular Ca cycling CRU network itself can give rise to many different patterns of SR Ca release^{23, 26}. We have aimed to further our understanding of these dynamical influences in this body of work.

In our DAD amplitude study in Chapter 5, we assessed the number of simultaneous Ca wave initiation sites as a determinant of DAD amplitude at the subcellular scale. The relationship we found can largely be explained intuitively. As the number of simultaneous wave initiation sites increases, total cytoplasmic Ca would increase within progressively shorter time periods and result in Ca transients and DADs of larger amplitudes and faster rates of rise. At the same time, multiple Ca waves would have less available time and excitable area on which to propagate within the myocyte and produce narrower Ca transient and DAD widths.

The mechanisms that govern the actual number of simultaneous Ca waves produced during an SR Ca release event are much less intuitive. In our spark-to-wave transition study in Chapter 2, we found that even when intracellular Ca load and spark frequency levels were sufficient, Ca waves did not initiate until Ca spark clusters of critical size formed within the myocyte. The nature of Ca spark cluster formation is random, in part, due to the stochastic nature of Ca sparks. At the same time, it is cooperative due to the diffusive Ca-mediated coupling of CRUs. The combined factors yield a critical phenomenon in which the transition from randomly firing Ca sparks to propagating Ca waves behave much like second-order phase
transitions underlying many real-world critical phenomena such as avalanches and earthquakes.\textsuperscript{28}

In our following study in Chapter 3 on the emergence of subcellular pacemaker sites and entrainment theory, we determined, through both experiments in real myocytes and simulations in our computational CRU network model, that Ca waves occur randomly in space and time when intracellular Ca is lower but more repeatedly from the same site when intracellular Ca is high.\textsuperscript{33} This wave entrainment behavior at high Ca levels was promoted by a shift to higher Ca wave initiation frequency and lesser variability at the whole-cell level than the average Ca release activity at the level of single CRUs. The sensitivity of this effect was heightened by dynamical heterogeneities as well as by molecular and structural heterogeneities, such as variations in the number of RyRs per CRU or reduced CRU spacing.

The multiple simultaneous Ca wave initiations we observed in our current study are likely a combined consequence of raising intracellular Ca levels to a regime in which Ca wave initiations are still random in space and time and increasing Ca cycling kinetics through $\beta$-adrenergic stimulation using ISO. Our observations in ventricular myocytes are consistent with those made in SA nodal cells under $\beta$-adrenergic stimulation.\textsuperscript{34}

6.1.2 Cellular-scale influences of arrhythmia trigger formation

The various patterns of spontaneous SR Ca release at the subcellular scale can directly influence DADs and EADs due to Ca-voltage coupling properties at the cellular scale. As discussed in our DAD amplitude study in Chapter 5, SR Ca load was studied as a major cellular scale determinant of DAD amplitude. The direct influences reported were fairly straightforward. As SR Ca content was raised, more Ca from the SR lumen was available for release into the cytoplasmic space over a given amount of time, thereby resulting in a proportional increase in DAD amplitude with little change in DAD duration. Higher SR Ca loads also facilitated
processes that further enhanced DAD formation, as with the number of simultaneous Ca wave initiations, which resulted in larger and narrower DADs as described in the previous section (6.1.1), and with the time course of SR Ca load refilling involved in shortening Ca release latencies and in enhancing the synchrony of spontaneous SR Ca release among cells at the tissue scale\textsuperscript{35}. As described in Chapter 3, higher SR Ca loads are also associated with the emergence of subcellular pacemaker sites and whole-cell Ca oscillations, which can contribute to increasing the frequency of DADs and TAs at the cell and tissue scales.

Dynamic feedback interactions among subcellular factors can also give rise to qualitatively new emergent properties and behaviors that are unique at the cellular scale. A common context in which these properties emerge is during the cardiac AP, in which voltage-gated ion channels simultaneously shape the same whole-cell voltage from which they are evolving. Alterations in the feedback relationship can result in arrhythmogenic triggers such as EADs, which are a product of a negative feedback relationship and time delay between inward Ca and outward K currents that produce oscillations in the AP plateau\textsuperscript{14}. As demonstrated in our study in Chapter 4, qualitatively new and complex features of EADs at the cellular scale emerged when the deterministic voltage-mediated processes underlying the most common form of EADs synergized with stochastic SR Ca release processes due to the property of Ca-voltage coupling within the myocyte.

6.1.3 Emergence of DADs at the tissue scale

For DADs to elicit a PVC or TA at the tissue scale, an estimated 800,000 contiguous myocytes in well-coupled homogeneous 3D tissue must develop Ca waves synchronously\textsuperscript{36}. As illustrated by a confocal imaging study of individual myocytes in intact ventricular tissue, sporadic Ca waves at slow heart rates were unable to generate DADs in tissue because adjacent myocytes without Ca waves acted as electrotonic current sinks that attenuated DAD
amplitude. As the degree of Ca overload progressively increased during rapid pacing, however, a critical mass of cells developed synchronous Ca waves sufficient to overcome the source-sink mismatch of neighboring myocytes and generate DADs\textsuperscript{31}. In order for synchronous Ca waves to develop in tissue, small variance in the timing of the onset of Ca waves is essential for effectively overcoming the source-sink mismatch.

Consistent with previous studies highlighting the significance of shortened latency and reduced variance in promoting DADs\textsuperscript{31, 35, 37}, our results suggest that Ca release synchrony in myocyte populations at the tissue scale, as determined by latency, is the most important factor that determines DAD amplitude. In essence, the latency period accounts for SR Ca release restitution mechanisms, which we believe include SR Ca refilling\textsuperscript{35}, CRU recovery from refractoriness\textsuperscript{37}, and criticality-based formation of Ca waves\textsuperscript{33}.

Previous studies in intact cardiac tissue have shown that Ca waves in individual myocytes generally do not propagate into neighboring myocytes but rather extinguish at cell borders or collide into other waves\textsuperscript{31, 36}. Yet, in well-coupled intact tissue, electrotonic current flows to minimize voltage differences between neighboring cells. These findings imply that though cell-to-cell coupling may only have a minimal effect on the synchronization of Ca release, there may be a substantial effect on the ability for DADs to trigger arrhythmias. This scenario brings into question the intriguing role that coupling among myocytes may play in mitigating the arrhythmogenic effects enhanced by Ca release synchronization in individual cells. Recent results from our modeling studies indicate that diminished cell-to-cell coupling among myocytes in tissue facilitate the dual role of DADs in generating arrhythmogenic triggers as well as a vulnerable tissue substrate\textsuperscript{38}.

6.1.4 EADs and DADs as causes of both trigger and tissue substrate vulnerability

According to the trigger-substrate hypothesis, an arrhythmia trigger must encounter a vulnerable tissue substrate in order for overt focal or reentrant arrhythmias, or both, to occur. In
most cases, the trigger and the substrate are considered to be independent factors that contribute to arrhythmia emergence. However, our more recent studies have demonstrated that the same dynamical mechanisms that promote trigger formation can also significantly contribute to the development of substrate vulnerability. In the case of EADs, our previous studies have shown that both the trigger and vulnerable substrate are made possible through a counter-balancing interplay between the divergent effects of dynamical chaos and the convergent effects of electrotonic coupling via gap junctions\textsuperscript{39}. This process of chaos synchronization serves to facilitate the ability for EAD-generating myocytes to produce PVC triggers at the tissue-scale by overcoming local suppressive source-sink effects by non-EAD-generating cells in well-coupled tissue\textsuperscript{32}. At the same time, the mechanism of chaos synchronization increases the degree of spatiotemporal APD dispersion across the tissue, which is a condition sufficient for enhancing tissue substrate vulnerability.

In the case of DADs, interplay between counteracting forces also exists in relation to the convergent electrotonic coupling effects in well-coupled tissue, but the divergent effects are attributed to the stochastic mechanism of criticality underlying Ca wave initiation and DADs. Accordingly, we have shown that DADs, which have also been classically considered as a major mechanism primarily for arrhythmia triggers, can also enhance the vulnerability of the tissue substrate. More specifically, our recent computer model simulations study\textsuperscript{22} demonstrates that “subthreshold” DADs, which do not reach the activation threshold for producing APs, are the major factor responsible for creating dispersions in refractoriness and excitability that lead to tissue substrate vulnerability. In this case, reduced Na channel availability, reduced coupling through gap junctions, increased tissue heterogeneity, and reduced synchrony in DAD latency can increase the probability of the simultaneous production of arrhythmia triggers as well as vulnerable tissue substrates.
6.2 Limitations

The limitations in our studies fall into three general categories. The first category pertains to limitations in experimental feasibility. In several of the experiments in the criticality study (Chapter 2), the pacemaker sites study (Chapter 3), and the complex EADs study (Chapter 4), ventricular myocytes had to be permeabilized in order to control intracellular Ca levels, which would normally vary due to the activity of membrane pumps and channels. Since the contributions of membrane activity were excluded, we were limited to demonstrating qualitative similarities but not strict numerical consistency in the behaviors exhibited between the experimental and virtual model systems. Under some circumstances, experiments were not feasible due to a lack of experimental means. For example, the role of structural heterogeneities could not be directly manipulated in real myocytes in the pacemaker sites study and the degree of gap junctional coupling could not be varied directly at the tissue scale in the DAD amplitude study (Chapter 5). The second category of limitations pertains to the feasibility of computational modeling. The most prominent issue was the inability to fully represent the complexities and details of the real myocyte in our detailed models. These include the fine structures of the real cell such as the t-tubule network and heterogeneities such as spatially non-homogeneous distribution and densities of ion channel expression, which could all potentially introduce novel dynamics and physiological behaviors. The third category of limitations pertains to the extent to which principles drawn from the specific context of ventricular myocytes in our studies can be extrapolated more generally to other cell types in the heart. Further studies would be necessary to determine whether Ca-mediated behaviors observed to be qualitatively similar among ventricular, atrial, and SA nodal myocytes are governed by the same mechanisms described in this body of work.
6.3 Clinical significance

SCD due to VF is the leading cause of death in the developed world and it affects the lives of more than 300,000 people in the U.S. alone, each year. Current clinical arrhythmia therapies, though effective within a specific range of conditions, have remained largely limited, warranting further investigation into the mechanisms underlying arrhythmias. As described in section 6.1, we have gained valuable insights into the biophysical and physiological mechanisms of arrhythmias through our body of work. Yet, we also intend for our studies to shed new light on avenues of arrhythmia therapy that target not only the function of individual molecules but also the dynamical behaviors that emerge from the collective interactions within the heart. In our previous experimental studies validating the dynamical basis of EADs, we have gained new insights into using genetic approaches to knock down auxiliary β subunits of LTCCs to more effectively suppress EADs than can current pharmacological approaches which mainly aim to block channels completely. From a dynamics perspective, this potential therapeutic approach is significant in that the strategy aims to suppress arrhythmias by modulating the timing of ion channel functions critical to dynamical processes than by decreasing the magnitude of currents essential for normal physiology. In like manner, the findings from the studies in this body of work suggest that new therapeutic approaches that aim to modify the 3R properties of CRUs within the CRU network may also be promising in suppressing DAD-mediated arrhythmias. One potentially promising strategy relevant to the findings in this body of work may be to decrease the degree of Ca-mediated recruitment of neighboring CRUs within the CRU network. Doing so would likely hinder the initiation and propagation of Ca waves as well as the formation of pacemaker sites and Ca oscillations at the subcellular scale. As a consequence, the probability of the formation of DADs and complex EADs at the cellular scale would likely decrease in turn, and the distribution of latencies at the tissue scale would broaden. Future studies would be required to validate the effectiveness of
the approach and also to identify the specific molecular targets that would diminish CRU recruitment while preserving normal Ca cycling and contractile function.

6.4 Future directions

Many questions and avenues of study remain yet to be pursued in future studies. In this current body of work, our main focus at the subcellular level has been on understanding the consequences on subcellular Ca release activity as the degree of interactions between CRUs was varied. However, less is known about the consequences of varying the degree of interactions within CRUs such as when the coupling between LTCCs and RyRs are varied. This scenario becomes relevant in understanding arrhythmias in the context of atrial and SA nodal myocytes, which lack the extensive t-tubule architecture that is present in ventricular myocytes. Understanding these consequences may shed new light into mechanisms of atrial arrhythmias, which although are less lethal than ventricular arrhythmias, occur much more prevalently. It may also reveal new insights into arrhythmia mechanisms involving abnormalities in the heart rate such as sick sinus syndrome, since the automaticity of SA nodal myocytes depends heavily on a Ca clock system that functions much like how Ca oscillations do in ventricular myocytes. Another context in which the consequences of decoupled CRUs can be investigated is in heart failure, in which t-tubule disruption decouples CRUs and leads to the appearance of orphaned RyRs. This decoupling has been associated with an increased prevalence of arrhythmias, reduced contractility, and Ca cycling defects. In our recent computational modeling study, we have investigated the consequences of t-tubule disruption and orphaned RyRs in light of the 3R theory and the rise of alternans. Direct experimental validation of results from the modeling study remains to be performed. In these contexts, it is intriguing to consider how CRU decoupling would alter the qualitative properties of criticality, Ca wave entrainment, the emergence of EADs with long plateaus, and latency distributions in these contexts.
Another avenue of study involves further exploration of factors investigated in the current body of work. In the DAD amplitude study (Chapter 5), we investigated specific multiscale determinants of DAD amplitude. Yet, directly identifying the dynamic relationships among factors at multiple scales will help better establish the understanding of the nature of multiscale processes in cardiac physiology, which still remains to be fully understood\textsuperscript{45}. The degree of gap junctional coupling has been investigated in our recent computational study as a major tissue-scale factor that promote both trigger formation as well as substrate vulnerability\textsuperscript{38}. Experimental validation of the findings in the whole-heart setting remains to be performed.

As mentioned briefly before, we intend to shed new light on therapeutic strategies for preventing and suppressing arrhythmias. In our current body of work, we focused heavily on the conceptual basis of how arrhythmogenic behaviors emerge dynamically from the collective interactions within the heart. To bring our findings closer to therapeutic application, specific molecular targets need to be identified so that they can be used to modulate dynamical factors of the system, such as the 3Rs, to non-arrhythmogenic regimes. In Chapter 5, we have identified the variability in the latency to the onset of Ca waves as the single most important factor for promoting DADs in the context of moderate Ca elevation and adrenergic stress. Identifying biological targets at the tissue scale that work to broaden latencies and desynchronize arrhythmogenic Ca release events may prove to be a promising strategy for suppressing DAD-mediated arrhythmias.

6.5. Concluding Remarks

As has been true throughout history and across civilizations, the understanding of the heart today has evolved in general accord with prevailing sets of beliefs. Today, genomics and molecular biology, and their diagnostic and therapeutic applications, have come to the forefront\textsuperscript{6} and have allowed biological problems to be broken down in far greater detail than has ever
been possible before. While advancing forward, the study of cardiac arrhythmias has found much success in breaking down large biological problems conceptually into component parts that can be solved separately. However, when piecing the parts together in drawing a comprehensive understanding of the nature of arrhythmias, the field of study has oftentimes encountered confounding and counterintuitive results not only within the realm of basic science\textsuperscript{46-48} but within the clinical research setting as well\textsuperscript{49, 50}. As many have found, the biology of the heart is immensely complex and the underlying basis for cardiac arrhythmias is intrinsically non-intuitive\textsuperscript{51}. In order to overcome some of the aspects of these challenges and to further unravel the inherent mysteries underlying cardiac arrhythmias, we have utilized a multidisciplinary approach that draws upon and integrates ideas from a diverse spectrum of fields, which primarily includes the application of nonlinear dynamics within the contexts of experimental electrophysiology and computational mathematical modeling. This approach has given us tremendous versatility in the way we could view, conceptualize, quantitatively assess, and logically predict the emergence of arrhythmias. The primary goal of our studies in this body of work has been to investigate the dynamics-based mechanisms by which the broad spectrum of spontaneous Ca release behaviors emerge from the CRU network and to understand the means by which these behaviors promote arrhythmias across the subcellular, cellular, and tissue scales of integration in the heart. Accordingly, we have discovered the role of a universal mechanism of criticality governing the transition from stochastic Ca sparks to propagating Ca waves, a general theory for the emergence of subcellular pacemaker sites for Ca waves and oscillations, the role of calcium-voltage coupling in linking the stochastic behavior of SR Ca release to the deterministic voltage-mediated relationships among membrane ion channels in giving rise to complex EAD morphologies, and the identification of the variability in Ca wave latencies as the most important tissue scale factor that promotes DADs in cardiac tissue exposed to moderate levels of Ca overload and adrenergic stress. In light of these findings, we
hope to have unlocked new avenues and perspectives for understanding cardiac arrhythmias and for further advancing therapeutic strategy and design.
Appendix
In our studies, we rely heavily on the concepts and tools from the study of nonlinear dynamics, which is a branch of study originating from the field of physics. Since this area of study may be unfamiliar to many in the cardiac arrhythmia field, an extended overview of the key principles that underpin our application of nonlinear dynamics and some of the unique contexts from which these principles have come forth have been outlined in the following.

A.1. Mathematics as a common language in nature

The application of mathematics in our studies is used as a language to model, describe, or represent biological processes in a way in which the functional relationships among the essential elements involved in a process of interest are framed within a logical context. The mathematical model and the assumptions used in its construction also essentially represent the hypothesis being tested. Therefore, the logical outcome of the model also represents the direct logical outcome of the hypothesis itself. This approach thus serves as a means to validate hypotheses and determine the extent to which the mechanisms underlying biological processes are understood. If, for example, the mathematical model is able to qualitatively recapitulate a biological process of interest as observed in reality, then the identification of the essential elements as well as the relationships among them has been sufficiently accurate. If there is also quantitative resemblance between the outcome and real observation, then the presumed degrees of relative influence of each element has been accurate as well.

The mathematics used here, in simple terms, is akin to how the application of mathematics has revolutionized the fields of physics and chemistry centuries earlier as apparent in the works of thinkers like Sir Isaac Newton. In these fields, representing concepts, ideas, and mechanistic processes as quantifiable variables within a framework of logical expressions has brought about distinct advantages over what could be understood with intuition alone. Among these advantages include the ability to predict both the qualitative behaviors and the quantitative
details of dynamically evolving systems. For example, the motion of a swinging pendulum of a
given length under a given gravitational constant can be described in its entirety by a single
expression relating the pendulum’s angular momentum to its angle of displacement. Since the
relationship among each of these factors is known, the unique position of any pendulum at a
given time can be determined if the initial values of the angular velocity and angle of
displacement are known. When the structure of the relationships among key variables within a
system is analogous across multiple systems, the qualitative characteristics of the systems are
also similar. For example, in a mass and spring system in which the mass is free to move
across an ideal frictionless surface along one dimension, the structure of the relationship
between the velocity of the mass with respect to its horizontal displacement is analogous to that
between angular velocity and angle of displacement in the pendulum system. Accordingly, the
mass and spring system is capable of exhibiting oscillatory motion as can the pendulum. If the
structure of the relationship among the key variables within a system exists across multiple
dimensions, the system would be capable of exhibiting more complex patterns of behavior that
yet retain similar qualitative characteristics. Such is the case with planetary motion as
envisioned by Galileo from his observations of pendulum motion. The orbits along which
planets revolve are a more complex pattern of motion when the system is viewed as a whole.
Yet, since the structure of the relationships among the key variables in planetary motion are
analogous to that of the pendulum, the oscillatory characteristic is preserved along each spatial
dimension of planetary orbit.

The predictability of system characteristics can be extended to various other scenarios.
These include the qualitative characteristics that arise in patterns of spatial structure, as
opposed to temporal patterns as discussed. The natural world is rife with examples such as
hurricanes, nautilus shells, and the human cochlea, which all form spiraling physical structures;
river tributaries, trees, and circulatory vasculature, which all form branches; and sand dunes,
tropical fish skin patterns, and the skin at human fingertips, which all form ridge or stripe-like
Figure A.1. Patterns in nature. Common dynamical processes may underlie the formation of spatial patterns conserved across multiple systems in nature.

patterns on the surface (Figure A.1). Similarities in the evolution of system behaviors, such as the physical phase changes of water, can also be predicted even among systems that do not share analogous structures of relationships among key variables. Sometimes, all that is required is a resemblance in the manner of change of the analogous variables between systems. Such is the idea established by Mitchell Feigenbaum during his process of deriving a theory for universality\textsuperscript{52, 53}. In his work, Feigenbaum discovered that the series of period doubling phenomena that populations in ecology undergo was not necessarily unique to the quadratic form of the logistic equation that Robert May used to model the populations\textsuperscript{54}. Feigenbaum found that a sine function within a specific range of values could also produce the same system behavior. What Feigenbaum eventually showed was that a system could undergo the series of
period doubling behaviors as long as the key variable in the system, regardless of its relationship structure, evolves in a unimodal fashion along a parameter of interest. Both the quadratic logistic equation and the sine function that Feigenbaum used, when graphed, share the common feature of producing smooth, concave-down curves with single maxima.

A.2. Mathematics as a language for biology

Within the last century, the application of mathematics has become increasingly prevalent in the complex and oftentimes unpredictable world of biology, owing much to the tremendous versatility in the way that biology can be viewed, conceptualized, and simplified using the language of mathematics. The year 1952, in particular, was a seminal year in which some of the most notable works in mathematical biology came to fruition. Among them include the published works of Alan Hodgkin and Andrew Huxley, which incorporated a detailed modeling approach to quantitatively describe electrical excitations in neurons. Their work won them the Nobel Prize and has become the foundation for the study of electrophysiology. In the same year, Alan Turing also published a groundbreaking paper which described morphogenesis from the perspective of it as a biological reaction-diffusion system in which biomolecular “morphogens” in simple non-equilibrium chemical reactions spontaneously form what we now refer to as Turing patterns in space and time. In contrast to the more biologically detailed modeling approach used by Hodgkin and Huxley, Turing utilized a more general approach by which he deduced that the structure of the negative feedback relationship shared between the morphogens in his system that act as short-range reaction activators and those that act as long-range reaction inhibitors was a common basis by which similar patterns would form in other types of reaction-diffusion systems. In fact, the patterns described by Turing had striking similarities to the spatiotemporal reaction-diffusion patterns oftentimes observed in the later-established Belousov-Zhabotinsky chemical reactions, the propagation of APs in the form...
of spiral waves and three-dimensional scroll waves in the arrhythmogenic heart\textsuperscript{52, 62}, and the structure of vasculature formed by immature mesenchymal cells during embryogenesis\textsuperscript{63}. Both of the approaches developed by Hodgkin and Huxley and Turing have become essential pillars of mathematical biology and have led to advancements across multiple fields including the development of detailed computational models of the cardiac AP\textsuperscript{64-66}, predictions of the spread of disease, and genetic regulation in the yeast cell cycle\textsuperscript{67}.

A.3. A brief primer on nonlinear dynamics

Nonlinear dynamics is a branch of study originally from the field of physics. By analyzing the structure of relationships among the key variables within a system, nonlinear dynamics asks and addresses the question of how a system as a whole evolves over time\textsuperscript{52}. In turn, it is able to provide very effective means for describing and predicting the many different possible behaviors that a system can convey. The ability to do so is tremendously important in biological systems because phenomena as common and familiar as protein-protein binding interactions, inhibitory feedback interactions, or any other process involving interference, cooperativity, or competition takes on the form of nonlinear equations in mathematical notation. Since nonlinear equations, unlike linear equations, cannot be broken down into parts to be solved separately and then recombined to arrive at the solution\textsuperscript{52} without contradicting the logical construct of the mathematical expressions, nonlinear systems can be very difficult, if not impossible, to solve using traditional analytical methods. Furthermore, these nonlinear relationships are what give rise to biological complexity. Without taking these relationships into account, the understanding of biological processes would not be complete or entirely accurate.

In nonlinear dynamics, biological systems are represented using systems of differential equations. In this context, each differential equation is dedicated to describing the manner in which one variable within the system evolves over the course of time as influenced by other
variables in the system and by non-variable parameters that directly modulate the extent to which the variable of interest influences the system. Accordingly, the number of differential equations within a system of equations depends on the number of variables essential to the biological system’s behavior of interest. In representing the many different scenarios in biological systems, differential equations can take on various forms. For linear systems, each representative variable within each differential equation is to the first power only (i.e. $x_1$). The variables for nonlinear systems, on the other hand, typically are expressed as products, powers, or functions (i.e. $x_1 x_2$, $x_1^3$, or $\sin x_1$). Differential equations can also account for the spatial dimensions over which variables act. For example, AP propagation across cardiac tissue can be represented using partial differential equations. If a variable of interest pertains to the entire system as a whole, such as the voltage of a single myocyte, the equation is expressed in terms of the simpler ordinary differential form. Furthermore, analytical interpretation of differential equations can yield important insights into the dynamical behaviors of biological systems. For example, the sign of the numerical value of a differential equation, which can simply be calculated for a certain point in time given the initial conditions of the system, indicates whether a variable is increasing when the sign is positive or decreasing when the sign is negative. When the numerical value of a differential equation is zero, the variable is undergoing no net change either due to dynamical equilibrium or stasis.

A point at which every single differential equation of a system has a numerical value of zero indicates a very special set of conditions around which the characteristic behaviors of a system can take shape. These special points within a system are called equilibrium points, and they are the cornerstones upon which system behaviors are determined. Equilibrium points have stability characteristics. Around stable equilibrium points, the numerical values of differential equations tend to converge such that the rate of change of the corresponding state variable slows dramatically. Around unstable equilibrium points, the numerical values of differential equations tend to diverge such that the rate of change of the corresponding state
variable accelerates. From the systems perspective, the range of possible system behaviors grows in number and complexity the greater the number of variables or dimensions in the system.

In analyzing these systems, the dynamical systems analysis approach relies on visualizing system behaviors qualitatively in terms of geometry and topology\(^{52}\) and by representing systems in graphical form in order to facilitate the ability to intuit and understand system behaviors. Accordingly, system behaviors, in nonlinear dynamics analysis, are represented as time-course trajectories in state space, which is an \(n\)-dimensional space representing the entire set of values of the \(n\) number of variables in the system. So, the state space for a one-variable system would be represented by a line, whereas the state space for a two-variable system would be represented by a plane. Among all the possible system values in state space, the specific trajectory is determined by the location and stability of equilibrium points as well as the initial conditions from which the system evolves. System behaviors, then, can be assessed according to the geometric patterns formed by trajectories. In the case of the pendulum, the trajectory for oscillatory motion would appear as a circle or a closed loop in state space defined by angular momentum on one axis and angle of displacement on the other. The trajectories for the oscillatory motion of the mass and spring system and the motion of orbiting planets would also appear in their respective state spaces as closed loops. Specific sets of values to which trajectories may sometimes converge are called attractors.

Dynamical systems oftentimes undergo sudden changes in qualitative behavior called bifurcations, which often underlie the many distinct and counterintuitive phenomena observed in system behavior. The series of period-doubling phenomena observed by Robert May and Mitchell Feigenbaum in their respective systems are examples of systems undergoing a sequence of bifurcations. In the biological world, the “molecular switch” phenomenon and the sudden electrophysiological bursting patterns of pancreatic islet cells are examples of biological systems undergoing dynamical bifurcations. From the nonlinear dynamics perspective,
bifurcations are caused by the emergence or disappearance of equilibrium points as the system evolves over time. Though variables determine the structure of relationships within a system and the presence of equilibrium points accordingly, bifurcations have much to do with how parameters, with their own dependencies on factors such as time, voltage, or ligand concentrations as observed in the biological world, are able to determine the degree of influence the variables can assert upon the system. Consider, for example, a nonlinear expression consisting of variables $A$, $B$, and $C$ and parameter $a$, as follows: $aAB+B+C$. If parameter $a$ should range in value from 0 to 1, the expression remains nonlinear and retains the form of $aAB+B+C$ for all values of $a$ greater than 0. However, when $a=0$, the expression simplifies to $B+C$, which has a linear form. In the systems context, this change in form constitutes a sudden qualitative change in behavior or a bifurcation. As was possible with trajectories in state space, bifurcations can also be visualized in graphical form by comparing a variable over the range of a parameter of interest. The now famous U-sequence was constructed in this way by May and Feigenbaum to visualize period-doubling bifurcations\textsuperscript{52}. For systems or variables with bifurcations that have multiple parameter dependencies, it is possible to construct parameter phase diagrams, which map system behaviors to regions of parameter combinations, much like the diagrams used in physics for indicating the solid, liquid, or gas states of water relative to pressure and temperature.

A.4. Dynamical chaos and stochastic behaviors

In 1963, Edward Lorenz published an intriguing paper describing an unusually complex and rich set of phenomena that emerged from a simplified three-dimensional model representing convection rolls in the atmosphere\textsuperscript{68}. This behavior was unique in that it was a system behavior that appeared to be completely random and complex when assessed over time yet counterintuitively emerged from a very simple set of deterministic rules in which no
stochastic elements were involved. What Lorenz described was a dynamical system behavior known as chaos, and it has since been shown to be a universal mechanism underlying countless deterministic systems in nature. Steven Strogatz defines chaos as an aperiodic long-term behavior in a deterministic system that exhibits sensitive dependence on initial conditions. In state space, chaotic regimes form strange attractors that have distinct geometries with fractal characteristics within confined boundaries. Dynamical chaos becomes relevant in our study of cardiac arrhythmias because our previous studies have shown that it is a major mechanistic basis underlying the formation of EADs in cells and tissue as well as the development of VF. It is important to contrast dynamical chaos from stochastic system behaviors, which represent truly random processes. It is interesting to note that in electrophysiological systems including cardiac myocytes and neurons, the characteristic behavior of single ion channels is stochastic in nature, yet the summated average behavior of all the ion channels within a system underlying the currents and voltage changes within a system can be described with deterministic equations by applying mean field theory. Although the deterministic and stochastic perspectives are conceptually distinct, both processes are involved in most natural systems in reality. In our body of work, we explore a unique context in which the stochastic dynamics of spontaneous SR Ca release usually responsible for DADs interact with the voltage-mediated deterministic mechanism responsible for EADs to give rise to novel and complex arrhythmogenic patterns and behaviors in the context of single cardiac myocytes.

A.5. Mathematical modeling strategies

Oftentimes, the dynamical equations used to described biological systems cannot readily be solved analytically. For these scenarios, the use of computers has been vital in being able to calculate representative equations and to graphically represent and understand system behaviors. In the field of cardiac biology, both of the approaches developed by Hodgkin and
Huxley and Turing have become vital. Following their pioneering work came new advancements by the likes of Denis Noble and Yoram Rudy, who made detailed computational models of the cardiac AP and cardiac electrophysiology\textsuperscript{64, 65, 70}. Since then, several approaches to modeling have been developed, each with its own strengths and weaknesses. In general, we use simplified models containing the most essential elements of the representative system in order to understand overall system behaviors much like the way Turing approached his study of morphogenesis. In turn, we use biologically detailed Hodgkin and Huxley-type AP models\textsuperscript{71} to relate the dynamical system behaviors to the actual molecular entities within the biological system. In our experience, utilizing both approaches has yielded the most comprehensive basis for understanding arrhythmia behaviors.
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