

Distribution Statement

Distribution A: Public Release.

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

Examination of Cholesterol Dependent Regulation of SUR2 in HL-1 Cardiomyocytes,
and Assessment of K_{ATP} Function and COVID-19 Related Drugs in hiPSC-
Cardiomyocytes

by

Robert M. Geiger

Dissertation submitted to the Faculty of the
Molecular and Cell Biology Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2021



UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814



**APPROVAL OF THE DOCTORAL DISSERTATION IN THE MOLECULAR & CELL BIOLOGY
GRADUATE PROGRAM**

Title of Thesis: "Examination of Cholesterol Dependent Regulation of SUR2 in HL-1 Cardiomyocytes, and Assessment of KATP Function and COVID-19 Related Drugs in hiPSC- Cardiomyocytes".

Name of Candidate: Robert Geiger
Doctor of Philosophy
March 22, 2021

THESIS AND ABSTRACT APPROVED:

DATE:

SNOW.ANDREW
.L.1400942230

Digitally signed by
SNOW.ANDREW.L.1400942230
Date: 2021.03.22 16:58:40
-04'00'

3/22/21

Dr. Andrew Snow
DEPARTMENT OF PHARMACOLOGY AND MOLECULAR THERAPEUTICS
Committee Chair

FLAGG.THOMAS.
P.1376882930

Digitally signed by
FLAGG.THOMAS.P.1376882930
Date: 2021.03.23 11:27:55 -04'00'

3/22/21

Dr. Thomas Flagg
DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS
Thesis Advisor

DEY.SAIBAL.
1239556791

Digitally signed by
DEY.SAIBAL.1239556791
Date: 2021.03.23 11:58:30
-04'00'

3/22/21

Dr. Saibal Dey
DEPARTMENT OF BIOCHEMISTRY & MOLECULAR BIOLOGY
Committee Member

BURNETT.BARRING
TON.G.1461039290

Digitally signed by
BURNETT.BARRINGTON.G.1461039290
Date: 2021.03.23 15:27:45 -04'00'

3/22/21

Dr. Barrington Burnett
DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS
Committee Member

HAIGNEY.MARK
.C.1026997611

Digitally signed by
HAIGNEY.MARK.C.1026997611
Date: 2021.03.25 08:08:27
-04'00'

3/22/21

Dr. Mark Haigney
DEPARTMENT OF MEDICINE
Committee Member

ACKNOWLEDGMENTS

I would like to thank my thesis advisor Thomas Flagg for the time he has spent mentoring me throughout these past six years. I learned a lot from you especially the importance of being able to communicate my data to others.

I would like to thank my committee members for their support during my thesis, especially Andrew Snow for continuing to be the chair of my committee despite also becoming an assistant dean for the graduate school.

I would like to thank Mark Haigney for the opportunity to work in his lab during the COVID-19 pandemic and explore the proarrhythmic characteristics of hydroxychloroquine and azithromycin.

I would like to thank Naheed Fatima for her guidance in learning to culture hiPSC-CM as well as her compassion for when my experiments failed.

I would like to thank Michael Klein for showing me the importance of using programming languages to analyze and present data.

I would like to thank James Schooley for his support in helping me navigate the bureaucracy of USUHS. Thank you for always finding ways to get me what I needed for my experiments, I really appreciate it.

Finally, I would like to thank my fiancé Guzal Khayrullina for all her support throughout these past 5 years.

DEDICATION

To my parents Bob and Susan. Thank you for providing me with so many opportunities throughout my life and showing me the importance of hard work and being kind to others.

COPYRIGHT STATEMENT

The author hereby certifies that the use of any copyrighted material in the dissertation manuscript entitled: Examination of Cholesterol Dependent Regulation of SUR2 in HL-1 Cardiomyocytes, and Assessment of K_{ATP} Function and COVID-19 Related Drugs in hiPSC-Cardiomyocytes is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.



Robert Geiger

May 15, 2021

DISCLAIMER

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

ABSTRACT

Examination of Cholesterol Dependent Regulation of SUR2 in HL-1 Cardiomyocytes and
Assessment of K_{ATP} in hiPSC-Cardiomyocytes

Robert Geiger, Doctor of Philosophy, 2021

Thesis directed by: Thomas P. Flagg, Ph.D., Associate Professor, Department of
Anatomy, Physiology and Genetics.

The ATP sensitive potassium channel (K_{ATP}) is a unique ion channel that links cellular metabolism to channel function. K_{ATP} is comprised of four pore forming Kir6.x subunits and 4 SURx regulatory subunits. The subunit isoforms are tissue specific and are thought to be regulated at the transcriptional level. While there have been extensive studies examining the structure and function of K_{ATP} , the regulatory mechanisms underlying subunit expression have not been fully characterized.

Previous studies demonstrated that histone deacetylase inhibitors (HDACi) increased SUR2 expression in a cholesterol sensitive manner. In Chapter II, we show a cholesterol-dependent mechanism regulating SUR2 expression. We find that Trichostatin A (TSA) reduces cellular cholesterol levels, while at the same time increasing SUR2 expression. Furthermore, TSA is observed to activate SREBP cleavage/activation and cause nuclear translocation, while exogenous cholesterol inhibits these effects. We show that activated sterol response element binding protein

(SREBP) significantly increases luciferase reporter gene expression driven by the upstream SUR2 promoter. Finally, we demonstrate that an SREBP inhibitor can reduce SUR2 expression. Taken together, we provide evidence that the cholesterol dependent regulation of SUR2 is mediated by SREBP. These results suggest that cholesterol homeostasis might affect cardiovascular K_{ATP} subunit expression .

We previously examined K_{ATP} subunit expression in HL-1 cardiomyocytes, however hiPSC-derived cardiomyocytes (hiPSC-CM) offer a more comparable model to humans. Several ion channels have been characterized in this model, however there is little evidence showing expression of functional K_{ATP} channels. In Chapter III, we assess hiPSC-CM for the presence of K_{ATP} . We show Kir6.2 protein expression throughout differentiation, while the SURx subunits are expressed later in the time course. Interestingly, while we show both SURx subunits are expressed, we demonstrate that P1075 a SUR2-specific channel opener reduces the field potential duration (FPD) more than diazoxide an SUR1 specific channel opener suggesting that SUR2 is the predominant isoform expressed in hiPSC-CM. Taken together, these studies reveal the presence of functional SUR2 specific K_{ATP} channels in hiPSC-CM.

Finally, as we were evaluating hiPSC-CM for K_{ATP} expression and function, the COVID -19 pandemic emerged. Several drugs were touted as having beneficial effects in fighting the virus. However, one drug combination, hydroxychloroquine and azithromycin, was concerning as each of these drugs individually can block cardiac ion channels, potentially leading to proarrhythmic effects to the heart. In Chapter IV, we examined the cardiovascular consequences of hydroxychloroquine/azithromycin using hiPSC-CM. We use multielectrode arrays (MEAs) to rapidly assess the effects of these drugs on hiPSC-CM. We observe a prolonged FPD with acute exposure, while chronic exposure results in several proarrhythmic markers including prolonged APD90 (90% repolarization), EADs and ectopy. Taken together, we demonstrate an approach that

quickly identifies the proarrhythmic characteristics of drugs that are abused or applied in unintended combinations.

TABLE OF CONTENTS

LIST OF FIGURES	xii
CHAPTER 1: Introduction	16
The ATP sensitive potassium channel.....	16
K _{ATP} in Pancreatic, Smooth Muscle and Cardiac Tissue	16
Overview of K _{ATP} Function	16
K _{ATP} in Pancreatic beta-cells	17
K _{ATP} in Smooth Muscle	18
K _{ATP} in Cardiac Tissue.....	18
K _{ATP} Structure	20
The Kir6.x Subunit.....	20
The Sulfonylurea Receptor (SURx) Subunit	22
Tissue Specific Assembly of K _{ATP}	23
Regulation of Subunit Transcription	26
Epigenetic regulation of K _{ATP} expression	26
hipsc derived cardiomyocytes	28
Applications of hiPSC-CM	29
hiPSC-CM are used as a model to study several diseases	29
Drug assessment	30
Ongoing coronavirus pandemic and opioid epidemic	32
Coronavirus Pandemic	32
Hydroxychloroquine and Azithromycin	32
Opioid epidemic.....	34
Loperamide	34
Hypothesis and specific aims	35
CHAPTER 2: Novel cholesterol-dependent regulation of cardiac K _{ATP} subunit expression revealed using histone deacetylase inhibitors	37
Abstract.....	38
Introduction.....	39
Materials and Methods	41
Cell Culture.....	41
Imaging Experiments	42
Protein Isolation and Western Blot Analysis	43
Qunitative <i>RT-PCR</i>	43
Luciferase Assay	44
Data analysis and statistics	44
Results	45
TSA treatment causes a decrease in Cellular Cholesterol and an Increase in SUR2 mRNA Expression	45
TSA Treatment Causes Cleavage of SREBP in HL-1 Cells.....	46

TSA Treatment Causes Trafficking of SREBP to the Nucleus of HL-1 Cells	47
SREBP Increases Expression from the SUR2 Promoter	47
The SREBP Inhibitor PF429242 Suppresses the TSA-induced Increase in SUR2 Expression	48
Discussion	48
Cholesterol and K _{ATP} in the Cardiovascular System	48
Cholesterol and Ion Channel Regulation.....	49
Cholesterol and Arrhythmia	50
HDACi – Dependent Depletion of Cholesterol	51
Conclusion.....	52
Acknowledgements /Author Contributions/ Disclosure	58
CHAPTER 4: Characterization of K _{ATP} in human iPSC - derived cardiomyocytes	59
Abstract.....	60
Introduction.....	61
Materials and methods	64
Cell lines and Culture Methods	64
Western Blot.....	64
hiPSC-CM Disassociation and Plating.....	65
Immunofluorescence	65
Flow cytometry.....	65
Analysis of K _{ATP} using multielectrode arrays.....	66
Data Analysis	67
Results	68
SUR1, SUR2 and Kir6.2 are expressed in hiPSC derived cardiomyocytes .	68
Subcellular Localization of K _{ATP} Subunits	68
P1075 activates the K _{ATP} channel more than Diazoxide	69
Discussion	70
SURx as the rate limiting subunit for K _{ATP} channel expression	70
hiPSC-CM express predominantly SUR2 K _{ATP} channels	70
Examination of SUR2A and SUR2B in hiPSC-CM	71
hiPSC-CM as a model to study disease causing mutations.....	72
Conclusion.....	72
Acknowledgements /Author Contributions/ Disclosure	77
CHAPTER 5: Rapid Assessment of Proarrhythmic Potential using Human iPSC-Derived Cardiomyocytes	78
Abstract.....	79
Introduction.....	80
Results	81
Discussion	83
Competing interests/Author Contributions/ Disclaimer	86
CHAPTER 6: Discussion	87

Major Findings	87
Regulation of K_{ATP} channel Biogenesis	87
hiPSC-CM as a model to study K_{ATP} function during metabolic stress	88
Comparison between Mature Adult Cardiomyocytes and hiPSC-CM	91
Assessing Drug induced Cardiotoxicity with hiPSC-CM yields comparable Results to Clinical Trials	92
Possible Mechanisms underlying FPD prolongation in hiPSC-CM	93
Closing	95
REFERENCES	96

LIST OF FIGURES

- Figure 1: Diagram representing the structure of the K_{ATP} channel. (A)** Shows the domains of each subunit and where they are located on the protein. **(B)** Shows the hetero octameric structure of the channel. Kir6.x subunits form the pore of the channel surrounded by four SURx subunits. Adapted from (63). 21
- Figure 2: Histone acetylation causes histones to interact more loosely to DNA, while removal of acetyl groups causes a tighter interaction.** HDAC inhibitors prevent the removal of acetyl groups, thus causing more histone acetylation and looser interaction with DNA. Adapted from (86). 27
- Figure 3: Relationship between an action potential, a field potential and an electrocardiogram. (A)** Diagram of an action potential with each phase labeled, measured from a single cardiomyocyte. The action potential duration (APD) is the time between phase 0 to phase 3. **(B)** Diagram of a field potential that can be measured from a monolayer of hiPSC-CM. The field potential duration (FPD) is the time between the Na⁺ depolarization spike and the K⁺ Repolarization wave **(C)** Diagram of an electrocardiogram (ECG) measured from a heart. The QT interval is the time between the QRS complex and the T-wave. 31
- Figure 4: TSA causes a time dependent reduction in cellular cholesterol and selectively increases SUR2 expression in a time and cholesterol sensitive manner. (A)** Representative micrographs of HL-1 cells following treatment with either TSA or TSA+Cholesterol for up to 72 Hrs. Cells were labeled with the fluorescent antibiotic filipin (0.05 mg/mL) to estimate cellular cholesterol concentration. **(B)** Summary analysis of filipin staining intensity normalized to control (0 Hr) showing the time-dependent decrease in cellular cholesterol following TSA treatment. (n=4 biological replicates, 14-61 cells per timepoint). Time course of relative SUR2 mRNA expression in HL-1 cardiomyocytes treated with TSA or TSA+Cholesterol for up to 72 Hrs. TSA increases the expression of SUR2 over time, and addition of exogenous cholesterol inhibits this effect. (n=3; *p*<0.05 ANOVA; Bonferroni Post hoc test **p*<0.05, 0 Hr vs. TSA 72 Hr). 53
- Figure 5: TSA treatment induces SREBP-1 cleavage and increases PCSK9, an SREBP dependent gene. (A)** Relative mRNA expression of Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) (n=4). **(B)** Relative SREBP-1 mRNA expression did not increase in HL-1 cells that were treated for 72 Hrs with TSA or cholesterol as indicated (n=4). **(C)** Representative western blot (anti-SREBP1; SC, cat # sc-365513) of whole cell lysates from HL-1 cells treated for 72 Hrs with TSA or cholesterol as indicated. Summary quantitation of cleaved SREBP-1 **(D)** and full length SREBP-1 **(E)** protein from blots as in (B). There was an increase in cleaved and concomitant decrease in full length SREBP-1 was only evident following treatment with

TSA. (n=5; $p<0.05$ ANOVA; Bonferroni Post hoc test $*p<0.05$ TSA vs control.)	54
Figure 6: TSA treatment induces nuclear translocation of SREBP-1. Images shown are representative Z-projections of HL-1 cardiomyocytes treated for 72 Hrs with TSA and cholesterol as indicated. Cells were labeled with DAPI (<i>left</i>) or anti-SREBP-1 (<i>right</i>) (Abcam, cat # ab28481). Nuclear staining of SREBP-1 was significantly greater in TSA treated cells compared to control or cells treated with both TSA and cholesterol. In each of the three experiments, 9 – 34 cells were analyzed for nuclear translocation. (n= 3; $p<0.05$ ANOVA; Bonferroni Post hoc test $*p<0.05$ TSA vs. Control).....	55
Figure 7: SREBP-1a can activate the SUR2 promoter. (A) 470 bp of the non-coding sequence immediately upstream of the SUR2 transcription start site were cloned into a <i>Gaussia</i> luciferase reporter vector. Analysis of this sequence revealed a putative SREBP binding site in the antisense strand (position -356 – -365 relative to the transcription start site) similar to that found in the PCSK9 gene (104). A mutant SUR2 sequence was constructed by deleting three nucleotides shown to be involved in SREBP binding (154). (B) Relative luciferase activity measured in COS-m6 cells transfected with luciferase reporter constructs. Co-transfection with SREBP-1a induced a significant increase in relative luciferase activity compared to co-transfection with vector control. Luciferase activity is not significantly increased when the putative SREBP binding site is mutated, while basal promoter activity (C) was significantly changed not by the mutation. (n=5-10, $*p<0.05$ Student's T-test.).....	56
Figure 8: The SREBP inhibitor PF429242 the effect of TSA on SUR2 expression. Relative mRNA expression in HL-1 cells treated with both TSA and different concentrations of the reversible competitive inhibitor of SREBP maturation, PF429242. Cells were treated with the inhibitor for the final 48 hours of the 72 hour treatment. (n=4-6; $p<0.05$ ANOVA; Bonferroni Post hoc test $*p<0.05$ TSA vs TSA+PF429242 75 μ M.).....	57
Figure 9: Induction of the SURx subunits correlates with Troponin T during Differentiation. (A) Shown are representative western blot results demonstrating the expression of K _{ATP} subunits (SUR1, SUR2, Kir6.2) and a marker of cardiomyocyte differentiation, troponin T(TnT). (B) Quantitation of results from blots as in A reveals a marked induction of SUR1 and SUR2 protein expression corresponding with cardiomyocyte differentiation and the appearance of TnT. (C) Troponin T levels were assessed with flow cytometry, cells stained anti – troponin T shown in the red histogram compared to unstained cells shown in blue. Representative confocal micrograph of a hiPSC-CM stained with anti – TnT (*, $p < 0.05$; ANOVA, n = 3)	73
Figure 10: Confocal images suggesting the SUR1 subunits may be retained in the ER. (A) Representative images of hiPSC-CM showing SUR1 may be retained in the ER and SUR2 in the plasma membrane. (B) Representative images showing subcellular localization of K _{ATP} channel proteins (SUR1, SUR2, Kir6.2) throughout the cell in hiPSC-CM. Given the inconsistent	

results, further experiments are needed in order to localize the K_{ATP} channel proteins. 74

Figure 11: FPD is shortened by the SUR2-selective agonist P1075 (A) (A)

Representative field potential recordings that have had part of the sodium spike removed to better view the T -waves and **(B)** summary analysis of FPD in hiPSC-CM (iCells²) monolayers treated with SUR2-selective K_{ATP} agonist, P1075. The FPD was measured from the “Na⁺ spike” to the peak of the “T wave”. P1075 induced marked shortening of the FPD, that was inhibited by glibenclamide (1 μ M). **(C)** While P1075 (SUR2 agonist) induces a marked concentration – dependant (IC₅₀ = 0.207 μ M) shortening of the FPD, the SUR1-selective K_{ATP} agonist had little or no effect of the FPD. (*, p < 0.05; ANOVA, n = 2-5). **(D)** Schematic showing the time between the cell depolarization spike and the repolarization T – Wave is the field potential duration (FPD). 75

Figure 12: APD90 is Reduced by the SUR2-Selective K⁺ Agonist, P1075. (A)

Shown are local extracellular action potentials (LEAP) recordings from hiPSC-CM monolayers paced at 1 Hz and treated with the indicated K_{ATP} agonists. The amplitude of the LEAPs were normalized to the maximum in each trace. **(B)**. Summary results demonstrating that P1075 causes a significant APD₃₀, 50, 90 shortening in a concentration dependent fashion, while diazoxide has little effect on APD₉₀. (*, p < 0.05; ANOVA, n = 2-5) ... 76

Figure 13: Evaluating proarrhythmic potential of loperamide using human iPSC-derived cardiomyocytes. (A)

Representative field potentials recorded from iPSC-derived cardiomyocyte monolayers before and 2-5 hours after loperamide exposure at the concentrations indicated. The field potential duration (FPD) was measured from the initial depolarizationspike (analogous to the QRS complex) to the end of the T-wave (as indicated by the vertical line). FPD was unaffected at therapeutic (3 nM) concentrations but markedly increased at supratherapeutic (300 nM) concentrations. Data points in the dose- response curve represent means $\pm$ SEM (n = 4-7). Curves represent the least squares fit to the data with a Hill equation (Hill coefficient = 1.3). **(B)** Shown are exemplar Local Extracellular Action Potentials (LEAPs) from the same cells recorded after 18-25 hours exposure to loperamide at the indicated concentrations. At supratherapeutic concentrations, LEAP recordings revealed frequent proarrhythmic electrical activity, including > 10% prolongation of the action potential duration (*red*), early afterdepolarizations (EADs; arrows, *blue bar*), and failure of stimulus capture (*green*). Bar-graph represents the proportion of wells exhibiting the indicated proarrhythmic phenotype (n = 3-4). 84

Figure 14: Evaluating proarrhythmic potential of hydroxychloroquine and azithromycin using human iPSC-derived cardiomyocytes. (A)

Representative field potential recordings illustrating FPD prolongation in cardiomyocytes exposed to increasing concentrations of hydroxychloroquine and azithromycin. Dose response curves represent the least squares fit of the data with a Hill equation (Hill coefficient = 1.2) (n =4-8). **(B)** Sample LEAP records from cells treated with either hydroxychloroquine,

azithromycin or both in combination. Summary results illustrate the frequency of monolayers exhibiting each identified proarrhythmic activity (EADs, *blue*; APD prolongation, *red*; loss of capture, *green*) with each of the treatments. APD prolongation and loss of stimulus capture were evident in cells treated with both hydroxychloroquine and azithromycin, however EADs were absent suggesting a complex interplay of the proarrhythmic consequences of the combined therapy (n = 3-8). All recordings were analyzed with the cardiac module of the Maestro Pro software or custom software programmed in Python or ID. 85

Figure 15: K⁺ Activation Reduces Cytoplasmic Ca²⁺ Transient Amplitude in hiPSC-CMs (A) Condensed recording illustrating the effects of K_{ATP} activation by P1075 on the amplitude of the Ca²⁺ transient. Each transient is the average of the three consecutive waveforms. (B) Representative calcium transients averaged from the last four wave forms of drug exposure. (C) Summary data showing the change in fluorescence after cells we exposed to each drug..... 89

CHAPTER 1: Introduction

THE ATP SENSITIVE POTASSIUM CHANNEL

The ATP sensitive potassium channel (K_{ATP}) discovered by Akinori Noma in cardiomyocytes represented the first ion channel shown to be directly inhibited by cytosolic ATP levels linking metabolism to ion channel function(184). Since that time, significant progress has been made towards identifying the structure. The channel is composed of both pore forming inward rectifier (Kir6.x) subunits and regulatory sulfonylurea receptor (SURx) subunits(105). These subunits and their corresponding isoforms are critical to the channel's tissue-specific physiological role. For example, in pancreatic beta cells (SUR1:Kir6.2) K_{ATP} activity controls insulin release, in smooth muscle (SUR2:Kir6.1) can regulate vascular tone and in cardiomyocytes (SUR2:Kir6.2), activation is shown to be protective during an ischemic event. Given the diversity of subunit isoforms expressed in these tissues and the different functional outcomes that arise because of them, an understanding of the mechanisms that regulate their expression is vital.

K_{ATP} in Pancreatic, Smooth Muscle and Cardiac Tissue

Overview of K_{ATP} Function

Potassium channels are generally classified into three classes: voltage/calcium gated channels, leak channels and inward rectifying channels(66). As described by the current voltage relationship, K_{ATP} is considered to be a weak inward rectifier. This class of channels pass current more easily in the inward direction, when the resting membrane potential is below E_K (the equilibrium potential for potassium as determined by the Nernst Equation), while channel activation at potentials above E_K result in a significantly smaller outward current. This inward rectification is thought to be an important energy saving

mechanism as the repolarizing outward K^+ current must be replenished by the Na^+/K^+ pump(66). The mechanism underlying inward rectification is due to voltage dependent block of outward by polyamine and magnesium(61, 96, 148, 156, 180). Polyamines are naturally occurring long alkyl chains containing two or more positively charged amino groups that bind to residues on the inner vestibule of the Kir6.x subunit, thus blocking outward K^+ current(150, 227).

Cellular ATP and ADP levels can regulate channel activity through binding to different sites on the K_{ATP} channel. Under normal cellular metabolism, ATP can bind to the Kir6.x subunit and inhibit K_{ATP} channel activity ($IC_{50} = 20-800\mu M$)(66). During ischemia or hypoxia, a reduction in cellular metabolism causes elevation of MgADP (100-500 μM) resulting in channel activation through binding to the **SulfonylUrea Receptor (SURx)**, even with ATP evoked inhibition, thus demonstrating that it is not just cellular ATP levels that regulate channel activity but the ADP/ATP ratio(48, 66, 137, 179, 184). There are also several pharmacologic potassium channel openers (KCO) that bind/activate K_{ATP} including pinacidil and diazoxide, while sulfonylureas bind/inhibit channel activity(66, 99, 196, 204). These activators require either MgADP binding or MgATP hydrolysis to stabilize the channel in the open conformation(23, 38, 47, 128). Activation of K_{ATP} causes an outward current that shortens the action potential duration (APD) resulting in less calcium entry (50, 129, 181, 264). As described below K_{ATP} channel activation results in different physiological outcomes depending on the tissue where it is expressed.

K_{ATP} in Pancreatic beta-cells

The role of the K_{ATP} channel in coupling cell metabolism with cell function is best characterized in pancreatic beta-cells. When blood glucose levels are high, cellular metabolism and ATP in pancreatic islet cells are elevated, resulting in K_{ATP} inhibition. This inhibition of K_{ATP} channels causes depolarization of the membrane, which in turn leads to

opening of voltage-gated Ca^{2+} channels causing Ca^{2+} influx, insulin release and a concomitant reduction in blood glucose levels. When blood glucose decreases, metabolism and cellular ATP are reduced. This causes K_{ATP} activation and hyperpolarization of the cell. That reduces calcium channel activation and insulin release(1, 2, 34, 163). This makes K_{ATP} a clinically important drug target, especially for patients with diabetes. Sulfonylureas, a class of drugs known to inhibit the channel, have been used since the 1950's to lower blood sugar by increasing insulin release(191, 198, 237).

K_{ATP} in Smooth Muscle

K_{ATP} is also found in smooth muscle, where it is shown to regulate vascular tone(177). Inhibition of K_{ATP} current results in cellular depolarization and activation of L- type calcium channels resulting in calcium entry, SR induced calcium release and vasoconstriction. In comparison, an increase in K_{ATP} current results in cellular hyperpolarization, decreased calcium entry and vasodilation(168, 169, 177, 234, 261)

The resting membrane potential in smooth muscle is -50mV and voltage gated calcium channels begin to be activated -40mV, as a result, K_{ATP} mediated hyperpolarization or depolarization of just a few millivolts can cause a dramatic change in vascular tone(176, 178). This makes K_{ATP} a clinically important target. Rogaine™ (minoxidil), a K_{ATP} channel activator is thought promote hair growth by dilating the blood vessels around the hair follicle causing increased blood flow and better hair growth(164). Activation of K_{ATP} in smooth muscle has also been shown to cause vasodilation and be an effective treatment for hypertension, although its effects on insulin signaling complicate its use clinically(103, 258).

K_{ATP} in Cardiac Tissue

The cardiac K_{ATP} channel is the most densely expressed potassium channel in the heart with an estimated 50,000 channels per cardiomyocyte(40, 179, 232, 233). As a result,

activation has a dramatic impact on shortening the action potential duration(40, 179). In fact only 1% of sarcolemmal K_{ATP} channels need to be activated in order to shorten the action potential by 50%(179, 223).

ATP sensitive potassium (K_{ATP}) channels are known to play a protective role in the heart during metabolic stress/ischemic events(63, 66). Channel activation causes reduced calcium entry, thereby decreasing the number of detrimental effects due to calcium overload including arrhythmia and excessive contraction that allows the cell to retain its ATP levels resulting in less cell death(7, 9, 119, 120, 138, 183) This protective mechanism is clinically relevant when a patient has a an ischemic event or “heart attack”.

The effects of K_{ATP} activation have best been illustrated in rodent models of ischemia-reperfusion injury(252). Ischemia is induced by stopping blood flow followed by reperfusion (abrupt restoration of media flow) resulting in an influx of calcium into the cell(113, 114). In this model, K_{ATP} is activated by ischemia causing a shortened APD. Therefore when the cells are reperfused there is less calcium influx resulting in less contraction allowing the cell to maintain its ATP levels(33, 162). Activation of K_{ATP} through ischemic preconditioning (IPC) is shown to be cardioprotective(80, 175, 202). IPC is induced by subjecting cardiac tissue to brief, intermittent periods of ischemia several times before exposure to ischemic reperfusion injury. These brief periods of ischemia deplete ATP just enough to activate the channel, therefore when the cells were exposed to longer ischemia, the channel was already activated enough to shorten the APD and protect the cell(81).

These findings demonstrate the variety of physiological functions that the K_{ATP} channel can regulate. One of the key differences between the channels in pancreatic beta cell and cardiomyocytes is the conditions for activation of the channel. In pancreatic cells, K_{ATP} is dynamically regulated in response to metabolic changes associated with varying levels of glucose in the blood. In comparison, cardiac K_{ATP} channels only respond to severe

metabolic inhibition such as ischemia. The structural makeup of the channel likely accounts for the different requirements of ATP for channel activation between tissues.

K_{ATP} Structure

The Kir6.x Subunit

The pore-forming subunit of K_{ATP} is a member of the Kir6 subfamily. There are two genes, *KCNJ8* and *KCNJ11*, that encode the protein isoforms Kir6.1 and Kir6.2 respectively(105, 107). Kir6.1 is expressed in vascular smooth muscle, whereas Kir6.2 is expressed in the pancreatic beta cells, neurons, skeletal muscle and cardiomyocytes(66). Within the K_{ATP} channel the Kir6.x subunit plays three critical functions: K⁺ selectivity, ATP binding and PIP2 activation.

One major function of the Kir6.x is to select for potassium ions. Kir6.1 and Kir6.2 are comprised of two helical transmembrane domains (TM1, TM2) with the amino and carboxyl group located in the cytoplasm. Located between these domains is the P-loop, that contains a “signature sequence” (T-I-G-[Y/F]-G) required for selection of potassium ions(90, 96). The Kir6.x subunit also contains sequences that regulate channel activity(251). Several amino acids have been suggested to be involved in ATP binding (R50, C166, I167, T171, K185) as they alter ATP sensitivity when mutated. However, no classical consensus ATP-binding motif has been identified(250).

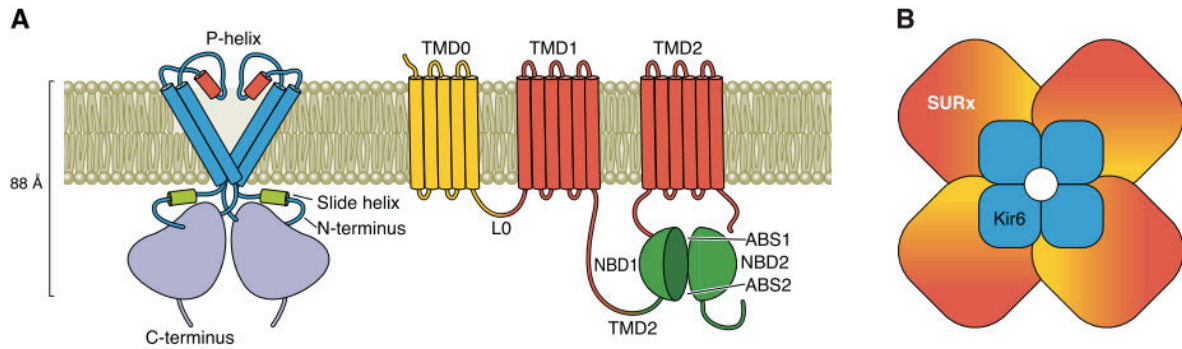


Figure 1: Diagram representing the structure of the K_{ATP} channel. (A) Shows the domains of each subunit and where they are located on the protein. **(B)** Shows the hetero octameric structure of the channel. Kir6.x subunits form the pore of the channel surrounded by four SURx subunits. Adapted from (63).

Stimulation of channel activity can also occur through binding of phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) (11, 54, 203, 228). PIP₂ binding increases the open probability of the channel by increasing the average channel open time and reducing the average channel closed time. This results in reduced channel activation by MgADP and channel inhibition by sulfonylureas (133). In addition, PIP₂ also reduces ATP sensitivity. This reduction is suggested to be a result of two mechanisms (66). First, the stabilization of the channel in the open confirmation decreases the ability of ATP to bind to Kir6.2 as the binding site is less accessible (51, 227, 249). Second, PIP₂ and ATP compete for the same binding site (52, 152, 203) as Kir6.2 residues R54, R176, R177, and R206 on Kir6.2 are thought to bind to PIP₂, suggesting that PIP₂ has a direct effect on ATP binding (11, 226, 228). Although less studied, Kir6.1 containing channels are also shown to be activated by PIP₂ (199).

In the absence of SURx, Kir6.2 is not expressed on the cell surface. Truncation of the COOH-terminal 26-36 amino acids of Kir6.2 was sufficient to enable trafficking of functional K_{ATP} channels to the plasma membrane (251). This confirmed that the Kir6.2 subunit harbors the ATP binding site for inhibiting channel function and led to the

identification of an RKR motif that binds to COPI vesicles causing subunit retention in the ER when exposed(98, 174, 275). Co-expression of the Kir6.x subunit with an accessory sulfonylurea receptor subunit (SURx) is normally required to mask this ER retention motif, enabling trafficking of the channel complex to the plasma membrane and functional expression of the channel(275).

The Sulfonylurea Receptor (SURx) Subunit

The SURx subunit is part of the ATP binding cassette (ABC) family of proteins. Like all ABC proteins, SURx proteins include two nucleotide binding folds NBF1 and NBF2 each containing a Walker A and Walker B motif forming the ATP binding sites(93). Magnesium (Mg^{2+}) bound to adenosine nucleotides (MgADP) binds to these NBF domains, activating the channel and causing an outward current, while ATP binds to Kir6.x inhibiting the channel (7, 15, 32, 40, 44, 52, 56, 57). MgATP can also activate channel activity although much less effectively than MgADP (79). Proteins in the ABC family typically function in transport through their transmembrane domains (TMD1,2). Interestingly, SUR proteins are somewhat unique among ABC proteins as no transport function has been determined for the SURx subunits.

The genes *Abcc8*, *Abcc9* encode SUR1 and SUR2 protein respectively(1, 31, 105). Interestingly, the subunits SUR1 and Kir6.2, in addition to SUR2 and Kir6.1, are located next to one another on chromosome 11p15.1, and chromosome 12p12.1 respectively, suggesting an undiscovered coregulatory mechanism between the two genes(31, 63, 106, 108). Due to alternative exon splicing, SUR2 can be expressed as one of two “full length” splice variants SUR2A including exon 39 and SUR2B including exon 40. These isoforms differ only in the COOH- terminal 42 amino acid residues(28, 66, 110, 224).

SUR2B and SUR1 containing K_{ATP} channels are more sensitive to activation by ADP. This is principally due to the sequence difference at the C-terminus of SUR2A. The terminal

42 amino acids produce an inhibitory effect on NBD2 mediated ADP activation of the channel making it more difficult for ADP to stabilize SUR2A channels in the open confirmation(157). This difference in ADP sensitivity explains why K_{ATP} is dynamically activated in pancreatic beta cells in response to changes in glucose, while in cardiac channels activation is rarely observed.

As the name suggests, SURx also binds to sulfonylureas, a class of drugs that inhibits the K_{ATP} channel. The binding site for sulfonylureas tolbutamide and glibenclamide is composed of the cytosolic loop in TMD0 near the N terminus and the cytosolic loop between TMD2 near the C terminus(165). SUR1 is more sensitive to sulfonylureas compared to SUR2(78). The SUR1 K_D for glibenclamide binding is $\sim 0.5 - 10\text{nM}$ (1, 45), while SUR2A and SUR2B have a binding K_D of $30 - 300\text{ nM}$ (45, 207, 208).

As mentioned earlier, K_{ATP} channels are also activated by pinacidil and diazoxide. The structural differences between the SUR2A splice variant and (SUR1 and SUR2B) account for the different pharmacological activators diazoxide and pinacidil/P1075(6, 157, 170, 253). Channels containing the SUR1 and SUR2B isoform are stimulated by diazoxide through binding to the TMD1 and NBD1 regions of the channel(6). In comparison, channels containing SUR2A and SUR2B splice variants are activated by P1075/pinacidil through binding to residues in the last helix of TMD2 region of the channel(64, 170, 197). The IC_{50} for diazoxide ranges from 0.1nM in atrial cardiomyocytes and 500uM in ventricular cardiomyocytes, while the IC_{50} for pinacidil ranges from 0.5uM in smooth muscle to greater than 500uM in pancreatic beta cells(66).

Tissue Specific Assembly of K_{ATP}

Kir6.x requires the SURx auxiliary subunit in order to generate a functional channel(105, 225). Initially, protein lysates from pig brain tissue were mixed a radioactive analogue of glibenclamide and boiled followed by SDS-PAGE. The results demonstrated that the sulfonylurea receptor (175 kDa) was expressed in the cerebral cortex of pigs(217).

Interestingly, a follow up study found that “omitting boiling” prior to SDS-page uncovered an additional 38 kDa protein that was also labeled by the glibenclamide analogue suggesting a tight linkage between the two proteins(218). Subsequent studies identified the 38 kDa protein as Kir6.2 and that when co purified with His-tagged SUR1, the resulting product was 950 kDa, almost the exact molecular weight of four SURx and four Kir6.x subunits, suggesting that this complex was an octamer. This was further corroborated in experiments showing that triple fusion constructs (SUR1-(Kir6.2)₂) could only generate functional channels when coexpressed with a functional SUR1 subunit(32). Taken together, this data demonstrates that K_{ATP} is composed of four inward rectifier (Kir6.x; *Kcnjx*) subunits that form the channel pore and four auxiliary sulfonylurea receptor (SURx; *Abccx*) subunits, combining in a 4:4 stoichiometry(32, 105, 225).

These subunits can assemble randomly to form functional channels in a heterologous system(25, 37). However, random subunit assembly is not often a phenomenon observed in nature as the subunit isoform expressed is homogenous in the tissue(63). For example, the SUR2A isoform is found mostly in cardiac and skeletal muscle, while SUR2B is expressed in smooth muscle(31, 66, 107). The SUR1 isoform is expressed in a variety of tissues including the brain, heart and most notably the pancreas(107). Given the variety tissues where K_{ATP} is expressed and the important role the channels play in regulating physiological function, several disease phenotypes can arise from subunit mutations.

In the pancreas, mutations in the channel subunits Kir6.2 or SUR1 cause impaired regulation of insulin secretion(182). Loss of function (LOF) mutations in either subunit account for the majority of persistent hyperinsulinemia hypoglycemia of infancy (PHHI). A disorder caused by an autosomal dominant mutation in SUR1(102). In fact, over 150 mutations in SUR1 and over 20 in Kir6.2 are known to cause the disease(65). Gain of function (GOF) mutations can lead to persistent channel activation resulting in permanent

neonatal diabetes mellitus (PNDM) a disorder caused by a autosomal dominant mutation in *KCNJ11* or *ABCC8*. (131, 139, 163, 215)

Gain-of-function (GOF) mutations in either SUR2 or Kir6.1 can cause K_{ATP} to be constitutively active resulting in Cantú syndrome(21). Patients with this disease exhibit hypertrichosis, craniofacial dysmorphism, patent ductus arteriosus, cardiomegaly, pericardial effusion, and lymphoedema(21, 183). Furthermore, Cantú patients are also demonstrated to have poor gut motility requiring a GI irrigation system for successful stooling(272).

Interestingly in the heart, Cantú patients are not observed to have a shortened APD. They exhibit slight changes in cardiac function such as increased ejection fraction and contractility(141). It has been suggested that Cantú patients exhibit increased L-type calcium current, thus prolonging the APD in order to compensate for the APD shortening mechanism caused by constitutively activated K_{ATP} (141).

Other studies examining cardioprotection have shown that overexpressing SUR2A can decrease myocardial infarct size compared to WT in mice exposed to I/R(47). Additionally, restoring Sur2 expression in a Sur2 null mouse was shown to increase resistance to ischemia and improve cardiac function during reperfusion compared to Sur2 null(236). In comparison, Kir6.2 null mice exhibit increased intracellular calcium with ischemia reperfusion injury compared to WT and in heterologous cell systems compared to cells transfected with K_{ATP} (9, 12, 119, 120, 241, 278). Furthermore, ischemic preconditioning failed to protect Kir6.2 null mice from I/R compared to WT(265).

Taken together, the above data illustrate the K_{ATP} channels are heteromultimers and that the subunits combine in a tissue specific manner to form functional channel complexes. Any combination of Kir6.x and SURx subunits can occur(25, 37), therefore it seems that the major factor for determining channel composition is subunit transcription. However, little is known about the molecular mechanisms that regulate K_{ATP} subunit transcription.

Regulation of Subunit Transcription

Several mechanisms including transcription factor (TF) activation or epigenetic modifications are implicated in regulating K_{ATP} subunit expression. Hypoxia and ischemia are shown to increase K_{ATP} subunit expression through the activation of different transcription factors(3, 35, 109, 221). The TF specificity protein 1 (SP1) was initially shown to increase SUR1 expression (91, 229). Later studies revealed that hypoxia inducible factor 1 alpha ($HIF1\alpha$) induced the expression of SP1 causing increased expression of SUR1(266). Another study suggested that forkhead TFs (FOX) play a role in mediating the response due to hypoxia/stress as K_{ATP} subunit expression correlated with increased FOX plasticity(192). Furthermore there are other putative binding sites for myocyte enhancement factor (MEF2) upstream of Kir6.1, Kir6.2 and SUR2, suggesting a possible role for MEF2 in K_{ATP} channel expression (193). These findings demonstrate that several transcription factors can regulate SURx expression, however we show that epigenetic regulation may also play a role.

Epigenetic regulation of K_{ATP} expression

Epigenetics is the study of changes in an organism caused by the modification of gene expression rather than alteration of the genetic code itself. For example, chemical modifications like histone acetylation can modify the accessibility of transcription factors **Figure 2** or DNA methylation can recruit suppressors and silence gene expression(85). We recently showed that SURx promoter methylation inversely correlated with expression in HL-1 cardiomyocytes suggesting methylation is involved with subunit regulation and demonstrating that epigenetic modifications could also regulate subunit expression(57).

Histone deacetylase inhibitors (HDACis) have been suggested to improve cardiac function in response to stress(159). Histone deacetylases or HDACs regulate gene

expression through the removal of acetyl groups from lysine residues on histones(85). This causes the histones to more tightly bind to the DNA as the histones are no longer shielded from the negative charges on the DNA, thus resulting in tighter DNA binding and less gene expression(160).

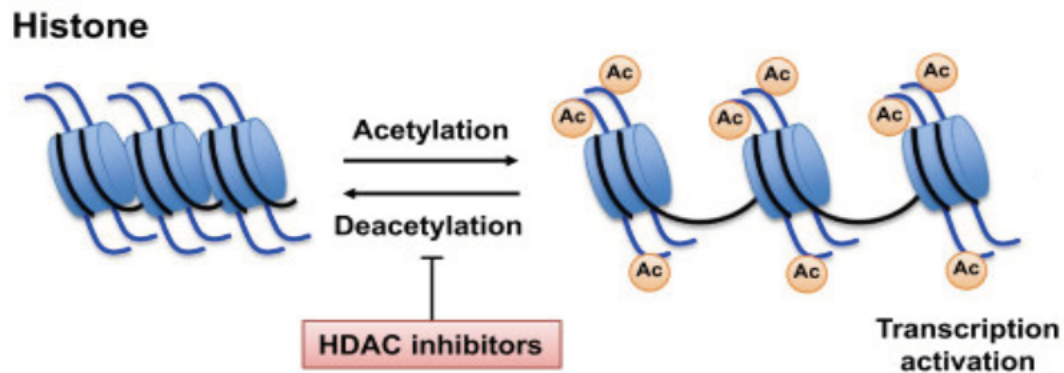


Figure 2: Histone acetylation causes histones to interact more loosely to DNA, while removal of acetyl groups causes a tighter interaction. HDAC inhibitors prevent the removal of acetyl groups, thus causing more histone acetylation and looser interaction with DNA. Adapted from(86).

HDACis have been used to treat a variety of diseases in the clinical setting. For example, HDACis are used in cancer treatment as they are suggested to upregulate genes involved in cell cycle arrest(62). However, there are also reports of HDACis affecting cardiac function. For example, in patients taking romidepsin, an HDAC inhibitor, for the treatment of cutaneous T-cell lymphoma (CTCL) have reported ST-segment elevation suggesting activation of K_{ATP} (185). Similarly, in a mouse model of ischemic reperfusion injury, trichostatin A (TSA), a pan-HDAC inhibitor demonstrated improved cardiac function, when compared to mice that were not treated with an HDACi (161, 276).

Recently, it was demonstrated that TSA increases SUR2 expression in HL-1 cardiomyocytes(56). Bioinformatic analysis demonstrated that 60/171 genes upregulated by TSA contained a putative sterol response element (SREBP) binding site in the promoter(56). This transcription factor is known to be activated by reduced levels of cellular

cholesterol(97). This data suggests that SUR2 may be regulated by a mechanism other than histone acetylation. In Chapter II we define a novel role for cholesterol in regulating SUR2 mRNA expression.

HIPSC DERIVED CARDIOMYOCYTES

Pluripotent derived cardiomyocytes were first demonstrated to be derived from embryoid bodies in 2001 using serum, yielding 5-10% cardiomyocytes(126). Since this time, improved protocols have been developed that can now generate a yield above 90% hiPSC-cardiomyocytes(147, 173). Most methods employ a small molecule-based approach. Briefly a GSK3 inhibitor (CHIR-99021) to induce mesodermal state, while subsequent Wnt inhibition with IWP2 initiates differentiation. One of the most efficient small molecule based methods uses heparin to improve differentiation(147). Furthermore, the base media in this method has been modified from the original B27 protocol to only contain the four necessary components (lipids, ascorbic acid, holotransferrin and selenium selenate) needed to differentiate hiPSC into cardiomyocytes. This helps to maintain consistent yields between batches(147). Unlike other cells, cardiomyocytes contain lactate dehydrogenase (LDH)(246). This enzyme converts lactate into pyruvate, thus allowing it be metabolized, while other cells starve as they do not have a carbon source. Therefore, lactate selection is commonly performed to ensure the highest yield of cardiomyocytes possible.

Previously, functional studies examining K_{ATP} have been conducted in various model organisms including heterologous cell systems, guinea pig, rat, mouse. These models have several benefits compared to humans; for example, their genome can be easily manipulated, and multiple organ systems can be examined at the same time. However, there are also notable differences, for example, their beat rate can be up to 10 times faster, and the repolarization of the AP is dependent on a different set of potassium channels. Within the past 10 years, cardiomyocytes derived from human inducible pluripotent stems

cells (hiPSC-CM) have emerged as a model system to study cardiac function. They share similar characteristics to mature adult cardiomyocytes(126, 274). hiPSC-CM have been used to study many ion channels including hERG and I_{Na} , yet there are few published examples examining K_{ATP} expression and function(151). Therefore, in Chapter 3, we set out to examine hiPSC-CM for functional K_{ATP} channels.

Applications of hiPSC-CM

Most hiPSC-CM protocols generate cells that exhibit a nodal, atrial or ventricular-like action potentials(151). These cells are spontaneously beating and share a similar beat rate to intact human cardiomyocytes at 30-40 bpm(151). There are two reasons underlying their spontaneous beat rate. Unlike mature adult cardiomyocytes, hiPSC-CM can initiate an action potential due to elevated amounts of funny current *HCN4* (I_f)(151). Furthermore, hiPSC-CM lack I_{K1} , a current critical to stabilizing the resting membrane potential to negative potentials, thus preventing an action potential and spontaneous beating from occurring(69). Given these characteristics, these cells are considered to be immature and possess a phenotype more similar to cardiomyocytes in the embryonic state(268). Despite these differences, hiPSC-CM have tremendous advantages over previous model organisms used to examine cardiac function. For example, cells used to generate hiPSC can be obtained in a minimally invasive way from a patient with a channelopathy. The hiPSC can then be differentiated into a potentially limitless supply of cardiomyocytes that exhibit the same genetic background as the donor patient. These cardiomyocytes can be used for various functional tests used to examine the effects of the mutations(125).

hiPSC-CM are used as a model to study several diseases

Ion channels including I_{Kr} , I_{Ks} , I_{Na} , I_{CaL} , I_f are demonstrated to be expressed in hiPSC-CM making these cells ideal for studying channelopathies(151). The examination of type

one long-qt syndrome was one of the first disorders to be examined in cardiomyocytes. hiPSC-CM were generated from a patient with a mutation in *KCNQ1*. Functional studies revealed a prolonged action potential duration and decreased I_{Ks} (134). Type 2 and 3 long qt syndrome caused by a loss of function mutation in I_{Kr} and a gain of function mutations in *SCN5A* respectively are also modeled in hiPSC-CM(244). Catecholaminergic polymorphic ventricular tachycardia (CPVT) was also shown to be modeled in hiPSC-CM through gain of function mutations in the ryanodine receptor (*RYR2*). Using this model, it was shown that dantrolene, a drug commonly used to treat malignant hyperthermia could also ameliorate the disease phenotype(121). After an ion channel is identified and characterized in hiPSC-CM, these cells can be used as models to study the disease phenotype associated with that channel. Therefore, identifying and characterizing K_{ATP} could lead to a model for studying Cantú syndrome.

Drug assessment

Because hiPSC-CM express many of the same ion channels found in mature adult cardiomyocytes, it is emerging as a good model for assessing drug toxicity. Multi-electrode arrays (MEAs) are high-throughput screens used to examine the electrophysiological changes in a monolayer of cells. These plates contain electrodes that measure small changes in ion concentrations as an action potential is conducted across a cell monolayer. The resulting measurement is known as a field potential. A field potential shares similarity to both an electrocardiogram (ECG) and the action potential (AP). It contains a sodium spike similar to both a QRS complex and phase 0 of an action potential, as well as a T wave similar to the repolarization wave in an ECG and phase 3 of an AP. The time between the field potential sodium spike and the peak of the T-wave is analogous to the action potential duration. We can measure FP to determine the APD in hiPSC-CMs (**Figure 3**).

Similar to FP, measurement of the local extracellular action potential (LEAP) can also be used to assess the APD. LEAP uses changes in voltage to induce strong coupling between groups of cells and electrodes within the plate allowing measurement of voltage changes arising in a cluster of hiPSC-CM (89). These signals resemble a cardiomyocyte AP and can be used to determine APD 30, 60 and 90. Furthermore, LEAP can also be used to assess any proarrhythmic markers caused by a drug like early after depolarizations (EADs), ectopy, or delayed after depolarizations (DADs). Taken together LEAP and field potential provide valuable insights into the electrophysiology of hiPSC-CM.

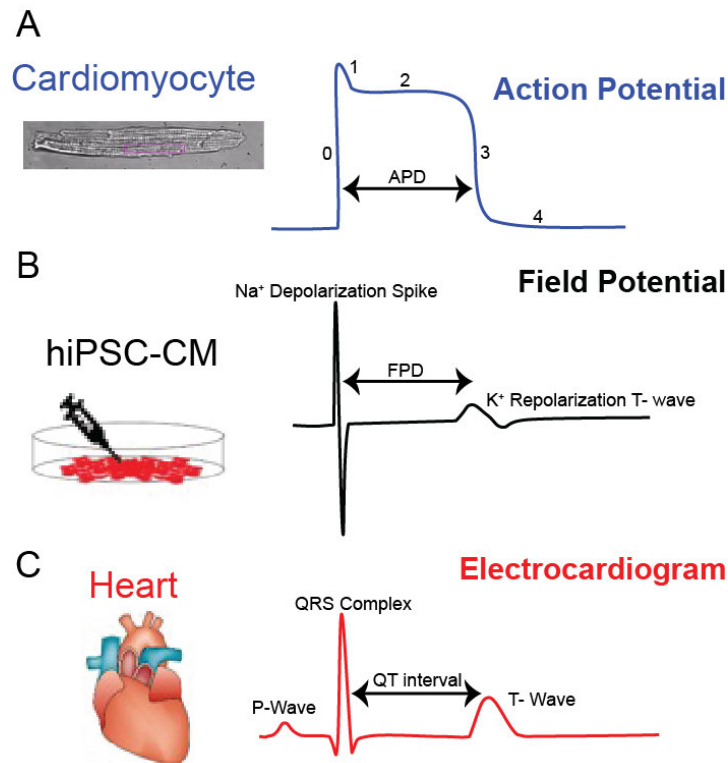


Figure 3: Relationship between an action potential, a field potential and an electrocardiogram. (A) Diagram of an action potential with each phase labeled, measured from a single cardiomyocyte. The action potential duration (APD) is the time between phase 0 to phase 3. (B) Diagram of a field potential that can be measured from a monolayer of hiPSC-CM. The field potential duration (FPD) is the time between the Na⁺ depolarization spike and the K⁺ Repolarization wave (C) Diagram of an electrocardiogram (ECG) measured from a heart. The QT interval is the time between the QRS complex and the T-wave.

ONGOING CORONAVIRUS PANDEMIC AND OPIOID EPIDEMIC

The rapid increase in SARS-CoV-2 infections has led to the current coronavirus pandemic. As a result, many drugs intended to treat a specific disease were quickly repurposed in an attempt to provide some level of care for infected patients. Two drugs, hydroxychloroquine and azithromycin were initially proposed to be an effective treatment option for patients with SARS-CoV2(73). Yet, little is known about their effectiveness in treating the virus and potential side-effects when taken together.

Before the corona virus pandemic started, another epidemic had already emerged as a public health threat. In the 1990s, many pharmaceutical companies began encouraging physicians to over-prescribe opiates for noncancer related pain causing a large number of people to become addicted, eventually leading to the current opioid epidemic(132). Although this is still ongoing, significant effort has been made to crackdown on opioid abuse causing addicts to look towards other drugs to get their high. Opioid dependent patients quickly discovered loperamide, an over-the-counter mu-opioid agonist used to treat diarrhea as an inexpensive alternative. Little is known about the drugs effect when taken at high dosages.

Cardiovascular arrhythmias are often complications when drugs are not being used as intended (loperamide) or are used in combination (hydroxychloroquine and azithromycin)(245). Furthermore, the ability to perform randomized control trials requires time that may be in short supply during a pandemic. In order to address these concerns, hiPSC-CM model system can be used to quickly identify any proarrhythmic characteristics these drugs may invoke on the heart.

Coronavirus Pandemic

Hydroxychloroquine and Azithromycin

Chloroquine and its clinically safer derivative hydroxychloroquine were originally developed during WWII as a prophylactic and treatment for malaria but now are commonly used to treat diseases lupus and rheumatoid arthritis(10, 123). However, in late 2019 when the virus was spreading in Wuhan China, a large effort was made to determine if pre-existing drugs were effective in treating the virus. Chloroquine, along with Remdesivir were among the initial drugs suggested to have antiviral effects(68, 262). Chloroquine was shown to reduce the glycosylation of the angiotensin converting enzyme 2 receptor (ACE-2), thereby reducing the amount of on the membrane of the cell and reducing the chance of infection(260). Furthermore, chloroquine can increase endosomal pH possibly interfering with viral fusion to the host membrane(262). A clinical trial conducted in France found hydroxychloroquine to be effective in reducing the viral load in COVID-19 patients(71). In the same study, azithromycin was also given with hydroxychloroquine to prevent a bacterial “super-infection”. Surprisingly, the treatment combination was found to be significantly more efficient at clearing the virus compared to hydroxychloroquine alone(71). Despite having a small sample size (48 patients), this study convinced people that the combination of drugs would be effective in treating COVID-19 patients. However, results from other clinical trials contradict these findings. A larger study (1446 patients) found that hydroxychloroquine alone did not reduce the risk of intubation or death(73). Most recently, a randomized clinical trial (667 patients) examined patients taking hydroxychloroquine with and without azithromycin, they found no improvement in clinical status compared to standard care(24). Although hydroxychloroquine and azithromycin are unlikely be an effective treatment for the COVID-19 patients, the drugs continued to be used to manage the virus given the publicity surrounding the treatment.

There are several cardiovascular consequences to consider when taking these drugs. Azithromycin and hydroxychloroquine are both demonstrated to block cardiac ion channels. Hydroxychloroquine can block potassium channels including the human cardiac

ether a` go-go-related gene (hERG), funny current (I_F), as well as L-type calcium channels(22, 123, 248). Azithromycin is shown to block sodium current (I_{Na}) with acute exposure, while chronic exposure increases I_{Na} , mild block of hERG and I_{Ks} were also observed(270). Blockage of potassium channels is known to prolong the action potential duration (APD) in a cell and the QT interval in a clinical electrocardiogram (ECG); both are arrhythmogenic markers. Interestingly, clinical studies have observed a prolonged QT interval in patients taking hydroxychloroquine, suggesting that the drug could be proarrhythmic(158). Although these drugs are suggested to exhibit proarrhythmic characteristics individually, little is known about their effects in the heart when taken together.

Opioid epidemic

Loperamide

As a result of the opioid epidemic, there was an increased effort to enforce new restrictions on prescription opiates leading many to look for alternatives. Loperamide (Imodium) was identified as a replacement as it is cheap and easy to access. Loperamide is a peripherally acting mu opioid receptor agonist developed in the 1970s for treating diarrhea(267). However, when consumed in excessive amounts can result in a euphoric high. According to a web-based study, the first mention of loperamide as an alternative to prescription opiates was in 2005 and since this time, the number of recorded exposures and deaths has continued to increase(256). Although the cause of death is still unknown, many people are reported to have had serious cardiac events(242). Several reports demonstrated that patients who overdosed from the drug also exhibited both a prolonged QT interval and widened QRS complex(259). Follow up studies revealed that loperamide can directly block specific ion channels in the heart suggesting a potential mechanism underlying the

phenotype found in patients who overdosed. For example, loperamide is demonstrated to block hERG causing a prolonged APD in cells or a prolonged QT interval in patients. Additionally, loperamide is demonstrated to block sodium current (I_{Na}), thus widening the QRS complex in the ECG(267). Taken together, consuming a supratherapeutic dose of loperamide can result in proarrhythmic markers leading to an increased risk of cardiac arrest and death.

HYPOTHESIS AND SPECIFIC AIMS

K_{ATP} is demonstrated to be involved in many physiological functions from regulating vascular tone in smooth muscle and insulin secretion in the pancreas to shortening APD in the heart. In the mouse model, the channel is shown to be involved in ischemic preconditioning suggesting a potential role in protection against ischemic reperfusion injury. While much is known about the channels function, little is known about how expression of the subunits is regulated. Furthermore, most of these functional studies were performed in the mouse model, a model known to have several physiological differences compared to humans. In order to investigate these questions....

1. We proposed to investigate the mechanisms regulating SUR2 expression in HL-1 cardiomyocytes. Previously we demonstrated that TSA depleted cellular cholesterol levels and upregulated SUR2 expression and that 60/171 genes upregulated by TSA had a putative SREBP binding site. Therefore, **we hypothesize that cellular cholesterol regulates SUR2 expression through an SREBP dependent mechanism.**
2. We proposed to investigate K_{ATP} expression and function in hiPSC-CM. Several functional ion channels are observed to be expressed in hiPSC-CM leading to the development of several disease models. Therefore, **we hypothesize that hiPSC-CM express functional K_{ATP} channels.**

As a result of the ongoing coronavirus pandemic, several drugs have been repurposed in an effort to provide relief for COVID-19 patients, while the opioid epidemic has caused prescription drugs to become more restricted leading many to abuse more accessible substances. Using these drugs in unintended ways can often lead to detrimental consequences to the heart. Clinical trials are the best way to identify unforeseen physiological outcomes however, they can take up to several months to complete. In order to quickly determine if these drugs could exhibit proarrhythmic characteristics...

1. We propose assessing the proarrhythmic characteristics of the drugs loperamide and hydroxychloroquine/azithromycin in combination on hiPSC-CM using a multielectrode array system. These drugs have previously been demonstrated to block several cardiac potassium channels. Block of these channels is known to prolong the QT-interval and APD, thus increasing the chance of an arrhythmia. Therefore, **we hypothesize that these drugs will exhibit proarrhythmic characteristics when used individually and in combination.**

CHAPTER 2: Novel cholesterol-dependent regulation of cardiac K_{ATP} subunit expression revealed using histone deacetylase inhibitors

Robert Geiger, Naheed Fatima, James F. Schooley, Jr., Jeremy Smyth, Mark C. Haigney*
and Thomas P. Flagg

Department of Anatomy, Physiology, and Genetics, Uniformed Services University for the
Health Sciences, Bethesda, MD, 20814

*Department of Medicine, Uniformed Services University for the Health Sciences, Bethesda,
MD, 20814

To whom correspondence should be addressed:

Thomas P. Flagg, PhD

Department of Anatomy, Physiology, and Genetics

Uniformed Services University for the Health Sciences

4301 Jones Bridge Road, Rm. C-2114

Bethesda, MD, 20814

Ph: (301) 295-3509

Fax: (301) 295-3566

e-mail: Thomas.Flagg@usuhs.edu

Running title: Cholesterol, SREBP and SUR2 Expression

ABSTRACT

We recently discovered that the histone deacetylase inhibitor, trichostatin A (TSA), increases expression of the sulfonylurea receptor 2 (SUR2; *Abcc9*) subunit of the ATP-sensitive K⁺ (K_{ATP}) channel in HL-1 cardiomyocytes. Interestingly, the increase in SUR2 was abolished with exogenous cholesterol, suggesting that cholesterol may regulate channel expression. In the present study, we tested the hypothesis that TSA increases SUR2 by depleting cholesterol and activating the sterol response element binding protein (SREBP) family of transcription factors. Treatment of HL-1 cardiomyocytes with TSA (30 ng/mL) caused a time dependent increase in SUR2 mRNA expression that correlates with the time course of cholesterol depletion assessed by filipin staining. This is specific for SUR2 and we observe little or no change in expression of the SUR1 subunit. Consistent with the cholesterol-dependent regulation of SREBP increasing SUR2 mRNA expression, we observe a significant increase in SREBP cleavage and translocation to the nucleus following TSA treatment that is inhibited by exogenous cholesterol. Further supporting the role of SREBP in mediating the effect of TSA on K_{ATP} subunit expression, SREBP1 significantly increased luciferase reporter gene expression driven by the upstream SUR2 promoter. Lastly, treatment with the SREBP inhibitor PF429242, significantly suppresses the effect of TSA on SUR2 gene expression in HL-1 cardiomyocytes. These results demonstrate that SREBP is an important regulator of K_{ATP} channel expression and suggest a novel method by which hypercholesterolemia may exert negative effects on the cardiovascular system, namely by suppressing expression of the K_{ATP} channel.

Key Words: *Abcc9*, SUR2, SREBP, trichostatin A

INTRODUCTION

It is estimated that about 30 million adults live with high serum cholesterol levels (≥ 240 mg/dL) (14). High cholesterol has been associated with poor cardiovascular health. Elevated serum cholesterol is correlated with atherosclerosis and significant morbidity and mortality (189); however, vascular pathology alone does not fully explain the benefits of cholesterol-lowering therapies in reducing the risk of developing fatal arrhythmias that originate in the heart (19). Studies have shown that a high cholesterol diet is associated with an increase in intercellular cholesterol in cardiac myocytes with effects on intracellular Ca^{2+} cycling (101, 112), but it is not entirely clear whether elevated cholesterol affects other aspects of cardiac physiology.

ATP sensitive potassium (K_{ATP}) channels are known to play an important role in the heart during metabolic stress (63, 66). K_{ATP} channels are activated when intracellular ATP concentration falls or MgADP rises (137, 179, 184). During myocardial ischemia, K_{ATP} activation serves to shorten the cardiac action potential, reduce Ca^{2+} entry into the cell and cause a characteristic ST-segment elevation in the ECG (9, 135, 144, 179). Inhibition of K_{ATP} with sulfonylureas or by genetic deletion of subunits causes increased myocardial damage following ischemia and reperfusion (33, 241). Conversely, increasing K_{ATP} activity using pinacidil or by overexpressing ion channel subunits is protective against ischemia reperfusion injury (33, 47). Sarcolemmal K_{ATP} channels are also essential for ischemic preconditioning where brief bouts of ischemia serve to protect the heart during a subsequent long-term ischemic event (80, 265). Given the importance of K_{ATP} in the response to cardiac stress, adjusting its expression is expected to have consequences on cardiovascular health.

In the mouse heart, the sarcolemmal K_{ATP} channel is a hetero-octomeric protein complex composed of four inward rectifier (*Kir6.2*; *Kcnj11*) pore-forming subunits combined with four sulfonylurea receptor subunits (*SURx*; *Abccx*) (30, 145, 240). In mouse atrial

cardiomyocytes, SUR1 (*Abcc8*) is the sulfonylurea receptor present, while in ventricular myocytes, SUR2A (*Abcc9*) is the major accessory subunit(30, 64). We recently showed that the canonical histone deacetylase inhibitor (HDACi), trichostatin A (TSA), causes a significant increase in the expression of SUR2 in HL-1 cardiomyocytes (56).

TSA is typically thought to increase gene expression by inhibiting histone deacetylases leading to an increase in histone acetylation that promotes unwinding of chromatin to increase transcription factors accessibility. In a previous study, we found a specific, TSA-dependent increase in histone H3 acetylation (Lys9) at the SUR2 promoter(56). Surprisingly, we also observed that cholesterol inhibited the effect of TSA on K_{ATP} subunit expression without affecting histone acetylation (56). TSA has been previously shown to reduce cholesterol accumulation in mutant Neimann Pick Type C1 (NPC) fibroblasts and SH-SY5Y cells (186, 194). Taken together, these previous results suggested that TSA depletes HL-1 cells of cholesterol and may simulate a cholesterol sensitive signaling pathway that regulates K_{ATP} channel expression.

A well-known cholesterol sensitive transcription factor is the sterol response element binding protein or SREBP (97). When cholesterol levels in the cell are sufficient, SREBP interacts with a protein complex containing sterol cleavage activating protein (SCAP) and Insig, sequestering SREBP in the endoplasmic reticulum (ER). When cholesterol levels in the cell fall, reduced cholesterol in the ER causes the SCAP/SREBP complex to dissociate from Insig and translocate to the Golgi, where SREBP can be cleaved by site-1 protease (S1P). Cleavage of SREBP allows the transcription factor to translocate to the nucleus, bind sterol response elements and activate transcription of target genes(263). Therefore, a decrease in intracellular cholesterol is predicted to activate SREBP signaling, while an increase in intracellular cholesterol inhibits SREBP-dependent gene transcription.

There are two different genes encoding SREBP isoforms—SREBP1 and SREBP2(17). SREBP1 has two splice variants (SREBP-1a and SREBP-1c) that are typically associated with regulating the genes required for fatty acid and cholesterol synthesis, while SREBP2 typically regulates expression of enzymes needed for cholesterol synthesis and transport. However, it is increasingly recognized that SREBP proteins can regulate the expression of genes other than those involved in cholesterol or lipid homeostasis. For example a recent study demonstrates a role for SREBP in regulating the parasympathetic response of chick cardiomyocytes through regulation of G-protein gated inward rectifying K⁺ channels expression (190). Interestingly, *in silico* analysis of the SUR2 promoter suggests that it contains a sterol response element (192), leading to the hypothesis that SREBP may be the cholesterol-sensitive mediator linking TSA treatment, cholesterol depletion and K_{ATP} subunit expression.

In this study, we tested the hypothesis that TSA increases SUR2 expression through a mechanism that requires cholesterol depletion and involves activation of SREBP signaling. The results show that increased SUR2 expression correlates with decreased levels of cellular cholesterol, cleavage and translocation of SREBP1. Moreover, we show that inhibition of SREBP signaling suppresses the effects of TSA on gene expression. Taken together, these results reveal a novel cholesterol dependent mechanism that regulates K_{ATP} subunit expression and may contribute to poor cardiovascular health associated with hypercholesterolemia.

MATERIALS AND METHODS

Cell Culture

All cells were maintained in a humidified incubator with 5% CO₂ at 37° C. HL-1 cardiomyocytes were cultured on tissue culture vessels coated with gelatin (0.02%w/v) and fibronectin (0.5%v/v)(Sigma) and were maintained in supplemented Claycomb media as previously described(49, 56, 57). COSm6 cells were maintained in Dulbecco's

Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum and (100ug/mL) Penicillin/Streptomycin. Cells were passaged using 0.05% trypsin/0.02% EDTA. In some experiments HL-1 cells were treated with Claycomb media supplemented with Trichostatin A (TSA), cholesterol (250x cholesterol lipid concentrate, Gibco cat # 12531-018) or PF429242 (Sigma cat # SML0667) at concentrations and durations indicated in the text.

Imaging Experiments

In all imaging experiments, HL-1 cardiomyocytes on glass coverslips were fixed with 4% paraformaldehyde (FD NeuroTechnologies, cat # PF101), quenched with 0.15% glycine and labeled for either cholesterol quantitation or SREBP-1 immunolocalization. Cells examined for cholesterol quantitation were stained with filipin (0.05 mg/mL; Cayman chemical, cat # 70440), PicoGreen (Invitrogen, cat # P11496), FBS 10% in PBS for 2 hours. Cells examined for SREBP-1 localization were blocked and permeabilized with 10% FBS, 0.1% Triton X-100 in PBS at RT) for 1Hr. Following blocking, cells were incubated with antibodies specific to SREBP-1 (1:50; Abcam, cat # ab28481) in 0.1% triton x100 at 4 degrees overnight. To visualize SREBP-1 antibody localization secondary Alexa Fluor 488 conjugated anti rabbit IgG 2° antibody (1:500 Invitrogen) with 0.1% triton x100 and DAPI in PBS at RT for 1Hr. Labelled coverslips were mounted with VECTASHIELD (Vector, cat # H-1000) on glass microscope slides and sealed with nail polish. To visualize filipin staining, cells were exposed to UV light (ex: 370-420 nM) and fluorescence (em: 410-460 nM) was collected on a Nikon Eclipse Ti2 microscope using a 40x oil objective. To visualize SREBP-1 localization, cells were excited at 495 nM and fluorescence was collected on a Nikon A1 resonant scanning confocal system. Image quantitation was performed on reconstructed Z-projections (0.5uM between each slice for the z stack) encompassing the entire volume of the cell. Images were quantitated

using Fiji and the mean fluorescence intensity was normalized to time 0 Hr and the nuclear fluorescence was normalized to control(216).

Protein Isolation and Western Blot Analysis

To isolate proteins, cells were lysed with RIPA buffer (Sigma, cat # R0278), supplemented with PMSF and protease inhibitors (Roche, cat # 11836153001). Protein concentration was estimated using the BCA assay (Thermo Scientific). Protein was separated using polyacrylamide gel electrophoresis (4-15%) and transferred to a PVDF membrane (Bio-Rad Inc.). The membrane was blocked in 5% milk and incubated in with anti-SREBP antibody (1:250; Santa Cruz, cat # sc-365513) overnight at 4 degrees followed by anti-mouse IgG secondary antibody (cat # A11008) in TBS-T for one hour. The signal was observed using SuperSignal West Dura Extended Duration substrate (Thermo Scientific) and images captured on the Bio-Rad ChemiDoc imager and analyzed using Fiji(216).

Quantitative *RT-PCR*

mRNA expression was assessed using qPCR as described previously (56). Briefly, RNA was extracted from HL-1 cells using Quick-RNA MiniPrep (Zymo Research) and treated with DNase to remove residual genomic DNA. RNA concentration and quality were determined using a NanoDrop 2000 Spectrophotometer (Nanodrop Technologies Inc.). cDNA was synthesized from 1ug of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems Inc.). qPCR was performed on a CFX384 Real-Time System C1000 Thermal Cycler (Bio-Rad Inc.) using Taqman probe and primer pairs obtained from Applied Biosystems, Inc. Reactions were performed in triplicate. Relative fold changes (normalized to *HPRT*) were reported as $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = \Delta Ct_{(drug)} - \Delta Ct_{(notreatment)}$.

Luciferase Assay

Transcriptional activation of the SUR2 upstream regulatory sequence by SREBP signaling was assessed using luciferase assays. Briefly, the 470 bp sequence immediately upstream of the SUR2 transcription start site was amplified by PCR and cloned into the pGluc Mini-TK *Gaussia* luciferase reporter vector (New England Biolabs; cat # N8086). cDNA encoding SREBP-1a (pcDNA3.1-2xFLAG-SREBP-1a) was a gift from Timothy Osbourne (Addgene plasmid #26801; http://n2t.net/addgene:26801;RRID:Addgene_26801)(247). A predicted sterol response element was identified in the SUR2 promoter using Genomatix software(23) and deleted three nucleotides (TCA) within the site using the Q5 site directed mutagenesis kit (New England BioLabs cat# E0554S) resulting in the construct pGluc SUR2_SRE_Mut-Luciferase. COSm6 cells were plated in a 24-well plate at a concentration of 50,000 cells per well. Following overnight incubation, each well was transfected with 500ng pcDNA3.1-2xFLAG-SREBP-1a and 500ng pGluc SUR2-Luciferase or pGluc SUR2_SRE_Mut-Luciferase using Lipofectamine 3000 (Thermo Scientific). Luciferase activity was assessed using the Pierce luciferase assay glow kit (Thermo Scientific), post-transfection. Total luciferase activity of each well was normalized to protein level. Protein concentration was determined using a BCA assay (Thermo Scientific).

Data analysis and statistics

Data was analyzed using GraphPad Prism software to determine significance between multiple treatments, we used a one-way ordinary ANOVA with a Bonferroni post hoc test. A students T-test was used to determine statistical significance between two groups. Unless otherwise stated, standard error of the mean is shown.

RESULTS

We previously showed that the canonical histone deacetylase inhibitor (HDACi), trichostatin A (TSA), causes a significant increase in expression of the SUR2 (*Abcc9*) subunit of the K_{ATP} channel. TSA treatment reduced cholesterol concentration in the cell and addition of cholesterol to the culture medium inhibits the effect of TSA without affecting histone acetylation (56). This result implied that there may be a cholesterol-sensitive mechanism activated by HDACis that is required for increased expression of SUR2 in addition to the transcriptional effects of increased histone acetylation. Therefore, we tested the hypothesis that TSA increases SUR2 expression through a mechanism that requires cholesterol depletion and activation of the sterol response element binding protein (SREBP) signaling pathway.

TSA treatment causes a decrease in Cellular Cholesterol and an Increase in SUR2 mRNA Expression

To elucidate the role of cholesterol in regulating SUR2 expression, we measured the time course of cellular cholesterol depletion (**Figure 4A,B**) and gene expression changes (**Figure 4B**) in TSA-treated HL-1 cardiomyocytes. As expected, TSA causes a significant increase in SUR2 mRNA expression that was correlated with a time dependent decrease in the cellular cholesterol concentration as estimated by labeling the cells with the fluorescent antibiotic, filipin(140).

Importantly, addition of cholesterol to the media prevented both the decrease in filipin fluorescence and the increase in SUR2 expression. The effect of supplemental cholesterol is specific for SUR2 as there is no significant change in SUR1 expression under the same conditions. Taken together, the temporal correlation of SUR2 expression with the decrease in filipin staining suggest that cholesterol depletion in the presence of TSA is the principal catalyst for changes in SUR2 expression and supports the

hypothesis that previously unrecognized cholesterol sensitive-mechanism may regulate K_{ATP} subunit expression.

TSA Treatment Causes Cleavage of SREBP in HL-1 Cells

SREBP is cholesterol sensitive transcription factor that is known to upregulate genes involved in cholesterol and fatty acid biosynthesis (97)(263)(17). Proprotein convertase subtilisin/kexin type-9 (PCSK9) is an important regulator of cholesterol uptake and is a well characterized SREBP-target gene (116)(118). In support of the hypothesis that TSA induces SREBP signaling, we observed a marked increase PCSK9 in TSA treated cells that was inhibited by the addition of cholesterol (**Figure 5A**). Therefore, we further investigated whether the SREBP signaling pathway was activated by TSA. Because histone acetylation is associated with increases of gene expression through epigenetic mechanisms, we first determined whether TSA increased SREBP expression. SREBP-1 mRNA was not increased (**Figure 5B**), therefore we next examined whether SREBP signaling was activated in TSA treated HL-1 cells. When cholesterol levels in the cell are sufficient, SREBP is localized to the endoplasmic reticulum. If cellular cholesterol falls, SREBP is trafficked to the Golgi, where it is cleaved to form the active SREBP transcription factor (263). Indeed, in western blots we observe a significant increase in the presence of the SREBP cleavage product (~ 68 kDa) indicating cleavage and a corresponding decrease in the amount of full length SREBP-1 in TSA treated cell lysates compared to control (**Figure 5C-E**). Moreover, when cells are treated with TSA and cholesterol, SREBP-1 cleavage is inhibited, consistent with the conclusion that the TSA-dependent fall of cellular cholesterol leads to cleavage and activation of SREBP signaling.

TSA Treatment Causes Trafficking of SREBP to the Nucleus of HL-1 Cells

Cleavage of SREBP frees the truncated protein so that it can traffic to the nucleus and bind sterol response elements in the genome (263, 271). Because TSA increases SREBP cleavage, it is expected that there will be more SREBP protein localized to the nucleus. To test this, we performed immunolocalization studies to determine whether TSA increased the amount of SREBP protein found in the nucleus (**Figure 6**). Indeed, HL-1 cells treated with TSA showed a significant increase in SREBP-1 localization to the nucleus when compared to either control cells or cells treated with both TSA and cholesterol. This result demonstrates that in addition to cleavage in the presence of TSA, SREBP is translocated to the nucleus where it can stimulate gene transcription.

SREBP Increases Expression from the SUR2 Promoter

We next sought to determine whether SREBP-1 can increase transcription from the SUR2 promoter. To accomplish this, we cloned approximately the first 500 bp of the noncoding region upstream of the SUR2 gene transcription start site into a plasmid encoding a *Gaussia* luciferase reporter gene. *In silico* analysis of this segment of the upstream regulatory region of the mouse SUR2 gene using Genomatix software (23) predicted an SREBP binding site, in agreement with similar data in human and rat (192) (**Figure 7A**). We also constructed a luciferase reporter construct where this predicted SREBP binding site was mutated. Co-transfection of pcDNA3.1-2xFLAG-SREBP-1a(247) with the luciferase reporter containing the SUR2 promoter causes a three-fold increase in luciferase activity (**Figure 7B**). However, co-transfection of SREBP-1a with the mutant luciferase reporter did not induce a significant increase in luciferase activity. Analysis of the basal promoter activity in both the WT and mutated constructs showed no difference in basal expression (**Figure 7C**). These results indicate that the SUR2

promoter responds to activation of the SREBP signaling pathway, suggesting that SUR2 is a novel SREBP target gene.

The SREBP Inhibitor PF429242 Suppresses the TSA-induced Increase in SUR2 Expression

Finally, to determine whether activation of SREBP signaling drives the increased SUR2 expression with TSA treatment, we used PF429242, a reversible competitive inhibitor that has previously been shown to reduce SREBP cleavage/maturation (88, 220). PF429242 significantly inhibited the effect of TSA on SUR2 mRNA expression in a concentration dependent manner (**Figure 8**), consistent with the conclusion that the increase in SUR2 expression following TSA treatment results from cholesterol depletion and SREBP activation.

DISCUSSION

The present studies show that histone deacetylase inhibitors (HDACi), like TSA, reduce cellular cholesterol activating a cholesterol-dependent mechanism that regulates expression of K_{ATP} channel subunits. Indeed, the data show that the TSA-induced increase in SUR2 gene expression correlates with cellular cholesterol levels and that SREBP is cleaved and translocated to the nucleus. Moreover, we show that suppression of SREBP signaling with PF429242 significantly reduces the TSA-dependent increase in SUR2 expression. When coupled with data from luciferase reporter assays, these results demonstrate that cardiac K_{ATP} subunit expression can be directly regulated by cholesterol-dependent activity of SREBP.

Cholesterol and K_{ATP} in the Cardiovascular System

Elevated serum cholesterol is associated with poor cardiovascular health(14, 189) and the development of hypertension(83). Most consequences of high cholesterol are attributed to the development of arterial atherosclerotic plaques with consequent

hypertension and increased risk for myocardial ischemia(189). Other findings associated with hypercholesterolemia are not as easily attributable directly to atherosclerosis and suggest that excess cholesterol may inhibit the function or expression of K_{ATP} channels. For example, hypercholesterolemia has been shown to impair ischemic preconditioning and the response to ischemia-reperfusion (36, 60, 75, 76, 130, 136, 153, 188, 243, 254), both of which require the sarcolemmal K_{ATP} (33, 47, 80, 241, 265). In addition, the cholesterol reducing drug, simvastatin, reduces the no-reflow area following myocardial infarction through a K_{ATP} -dependent mechanism with an increase in SUR2 expression(269). Here we present a possible explanation for this finding, where reduction of cellular cholesterol activates SREBP signaling and increases K_{ATP} subunit expression. The present study is focused on cardiomyocytes, however SUR2 is also a component of K_{ATP} channels in vascular smooth muscle(29, 172, 273). Given the importance of K_{ATP} in regulating blood pressure(29, 100, 143, 166), it is intriguing to speculate that cholesterol dependent reduction of SUR2B expression in vascular smooth muscle contributes to the hypertensive phenotype associated with hypercholesterolemia.

Cholesterol and Ion Channel Regulation

Cholesterol is a critical component lipid of the cell membrane and there have been a number of studies examining the role of cholesterol in modulating ion channel function(142). For example, cholesterol loading inhibits recombinant Kir2.1 channels expressed heterologously in CHO cells, while cholesterol depletion increases current(206). This appears to be due to direct interaction of cholesterol with residues in the cytoplasmic domain of the channel(53). Studies also show that targeting to cholesterol-enriched membrane domains is critical for normal regulation of K_{ATP} channel function by protein kinase A(211). In the present study, we propose that cholesterol regulates ion channel subunit transcription through SREBP signaling. This is analogous

to studies showing that Kir3.1 expression is increased in chick cardiomyocytes incubated in lipoprotein-depleted medium as a result of SREBP activation(190). SREBP has principally been associated with regulating the genes required for fatty acid and cholesterol synthesis and transport, however it is increasingly recognized that the importance of SREBP is not limited to lipid homeostasis(115, 222). Indeed, the results presented here add to this growing body of literature linking cholesterol and SREBP signaling with non-metabolic physiological outcomes.

Cholesterol and Arrhythmia

Increased K_{ATP} activity accelerates repolarization, reduces the refractory period and predisposes to re-entrant arrhythmias(15). It was recently demonstrated that trigger induced ventricular tachycardia or fibrillation during ischemia was significantly reduced in hypercholesterolemic mice ($Ldlr^{-/-}$ or $ApoA1^{-/-}$)(4). This result of that study is consistent with the present study indicating that cholesterol suppresses K_{ATP} expression by inhibiting SREBP. Countering this observation, treatment with cholesterol-lowering statins is associated with reduced arrhythmia risk(19). K_{ATP} channel activation can also stabilize the membrane potential and prevent triggered arrhythmias. It is possible that statin-induced cholesterol reduction increases K_{ATP} expression to prevent arrhythmias, however it is difficult to discern since statins are known to have pleiotropic effects variously attributed to modulation of nitric oxide synthase, Rho/ROCK kinase, Rac, and the transcription factor peroxisome proliferator activated receptor (PPARs)(187). Importantly, statins may affect other ion channels as well. For example, simvastatin has been shown to partially reverse the reduction in Kv4.3 expression in hypertrophic cardiomyocytes (238). Nevertheless, the observation that K_{ATP} expression can be regulated by cholesterol and SREBP signaling suggests another way in which hypercholesterolemia and statin therapy may affect the heart.

HDACi – Dependent Depletion of Cholesterol

TSA is a canonical HDACi that is known to increase histone acetylation generally and in HL-1 cardiomyocytes specifically(56). In the present study, we show that TSA causes a time dependent decrease in intracellular cholesterol. TSA has been previously shown to reduce cholesterol accumulation in mutant Neimann Pick Type C1 (NPC) fibroblasts and SH-SY5Y cells(186, 194). The mechanism underlying cholesterol depletion is not clear. In SH-SY5Y cells, it has been proposed that TSA modulates expression of cholesterol trafficking genes(186). In addition, treatment of HepG2 cells with TSA causes reduced expression of 11 of the 15 genes involved in cholesterol biosynthesis suggesting an activation of SREBP signaling(26). It remains unknown why TSA causes a reduction in cholesterol in HL-1 cardiomyocytes. In principle, TSA may either increase cholesterol export, reduce cholesterol synthesis, or reduce cholesterol uptake. Interestingly, we do observe a significant (41-fold) upregulation of PCSK9 with TSA treatment. PCSK9 is a protease that serves as a chaperone for the LDLr causing it to be recycled from the plasma membrane and targeted for degradation(118). The use of PCSK9 inhibitors represents a new approach to lower cholesterol in patients with hypercholesterolemia, achieving significant reductions in circulating cholesterol by stabilizing LDLr in the hepatocyte membrane and increasing cholesterol uptake(209). It remains unknown how TSA causes cholesterol depletion in HL-1 cells and additional studies will be needed to examine this interesting observation. Nevertheless, in the context of the present study, TSA is a potent molecular tool to deplete cellular cholesterol and reveals a novel cholesterol-dependent mechanism of K_{ATP} subunit expression.

Conclusion

In summary, we demonstrate that TSA causes a reduction in cellular cholesterol that activates SREBP signaling and increases SUR2 expression. It is known that aggressive cholesterol-lowering therapies have significant benefit for patients with cardiovascular disease. We postulate that cholesterol lowering therapies would increase K_{ATP} as one of the many beneficial effects on the cardiovascular system. Moreover, because K_{ATP} channels protect the heart during metabolic stress, patients with hypercholesterolemia may not only be both more likely to experience an ischemic event due to atherosclerosis, but less able to cope with the ischemic event due to reduced K_{ATP} expression in the heart.

Figures

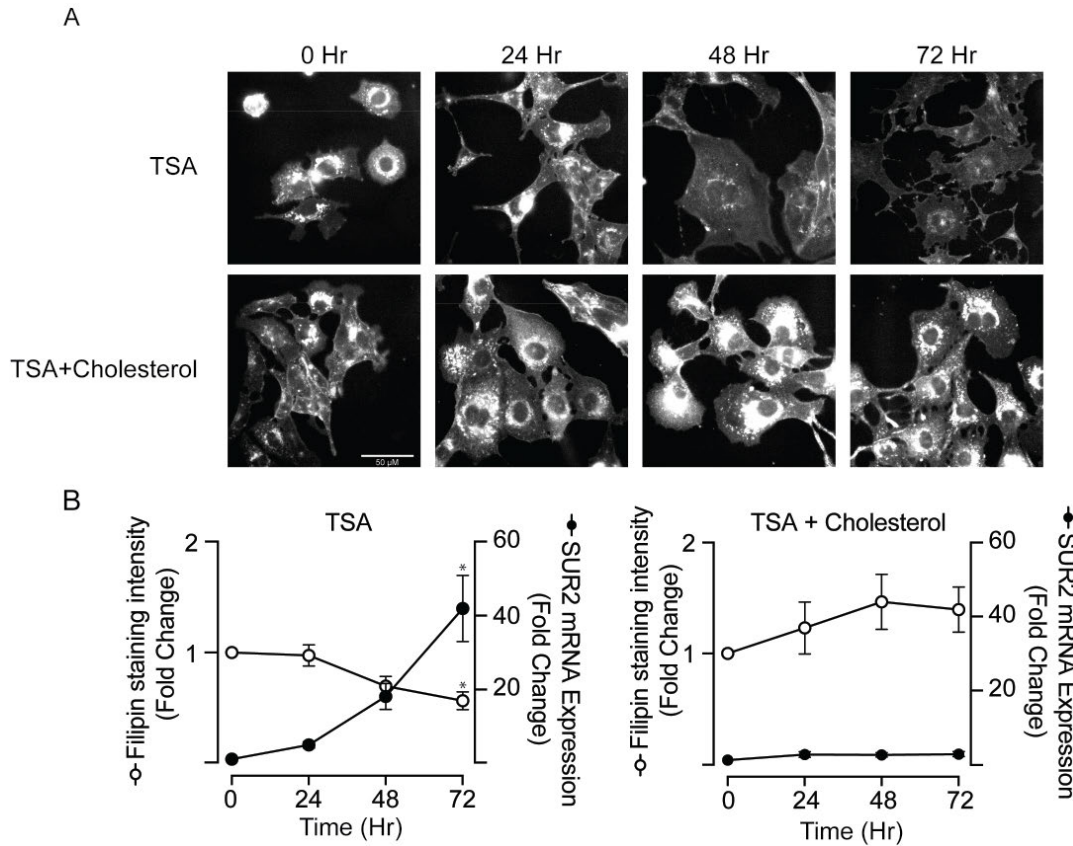


Figure 4: TSA causes a time dependent reduction in cellular cholesterol and selectively increases SUR2 expression in a time and cholesterol sensitive manner. (A) Representative micrographs of HL-1 cells following treatment with either TSA or TSA+Cholesterol for up to 72 Hrs. Cells were labeled with the fluorescent antibiotic filipin (0.05 mg/mL) to estimate cellular cholesterol concentration. (B) Summary analysis of filipin staining intensity normalized to control (0 Hr) showing the time-dependent decrease in cellular cholesterol following TSA treatment. (n=4 biological replicates, 14-61 cells per timepoint). Time course of relative SUR2 mRNA expression in HL-1 cardiomyocytes treated with TSA or TSA+Cholesterol for up to 72 Hrs. TSA increases the expression of SUR2 over time, and addition of exogenous cholesterol inhibits this effect. (n=3; $p < 0.05$ ANOVA; Bonferroni Post hoc test $*p < 0.05$, 0 Hr vs. TSA 72 Hr).

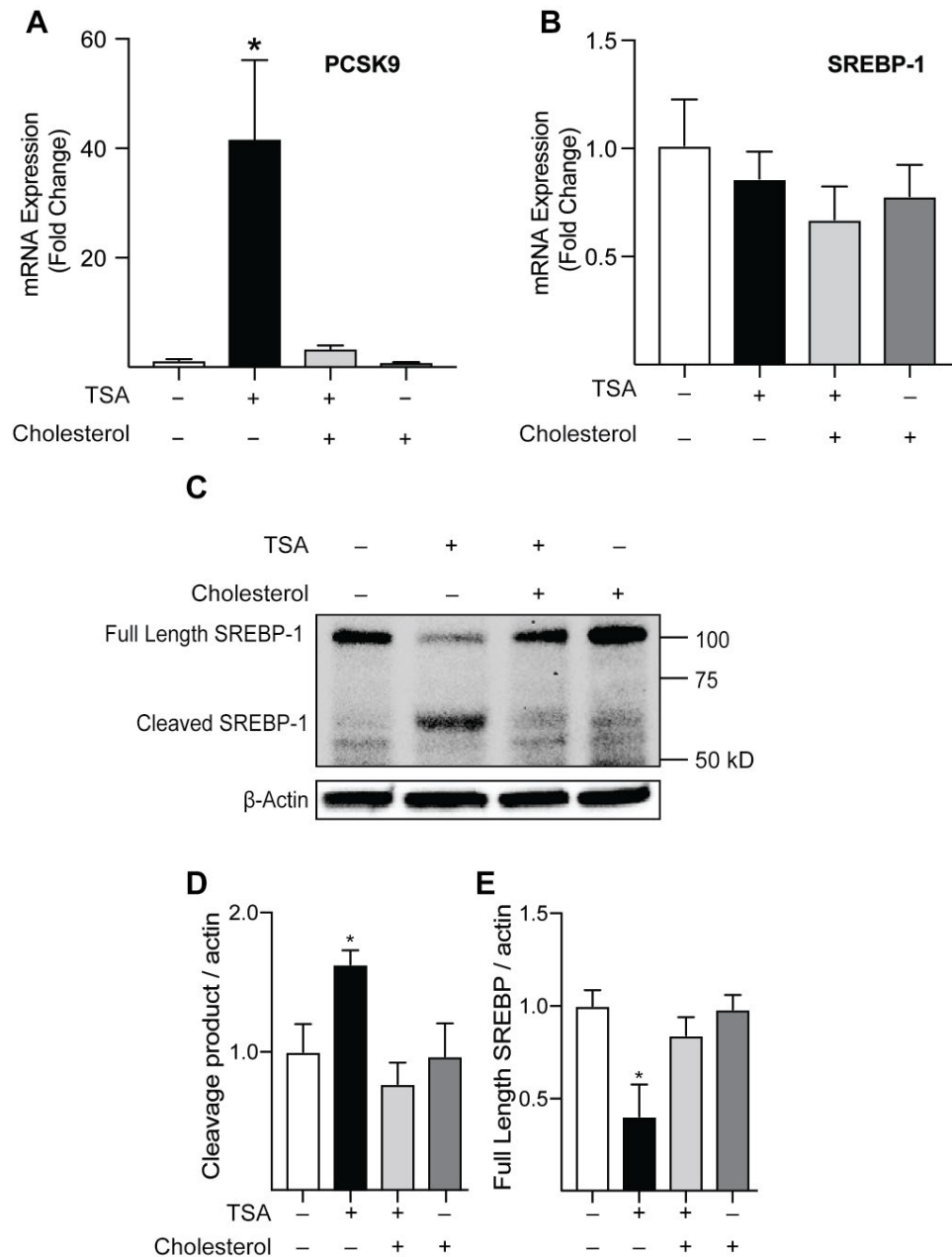


Figure 5: TSA treatment induces SREBP-1 cleavage and increases PCSK9, an SREBP dependent gene. (A) Relative mRNA expression of Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) (n=4). **(B)** Relative SREBP-1 mRNA expression did not increase in HL-1 cells that were treated for 72 Hrs with TSA or cholesterol as indicated (n=4). **(C)** Representative western blot (anti-SREBP1; SC, cat # sc-365513) of whole cell lysates from HL-1 cells treated for 72 Hrs with TSA or cholesterol as indicated. Summary quantitation of cleaved SREBP-1 **(D)** and full length SREBP-1 **(E)** protein from blots as in (B). There was an increase in cleaved and concomitant decrease in full length SREBP-1 was only evident following treatment with TSA. (n=5; $p < 0.05$ ANOVA; Bonferroni Post hoc test $*p < 0.05$ TSA vs control.)

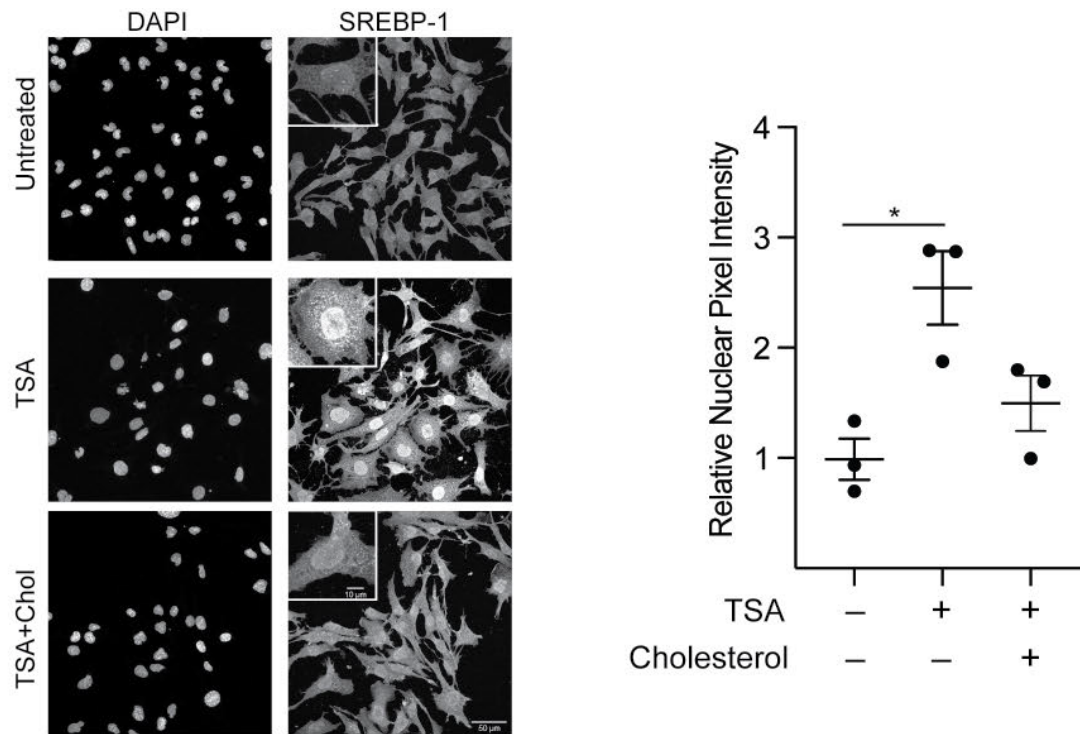


Figure 6: TSA treatment induces nuclear translocation of SREBP-1. Images shown are representative Z-projections of HL-1 cardiomyocytes treated for 72 Hrs with TSA and cholesterol as indicated. Cells were labeled with DAPI (*left*) or anti-SREBP-1 (*right*) (Abcam, cat # ab28481). Nuclear staining of SREBP-1 was significantly greater in TSA treated cells compared to control or cells treated with both TSA and cholesterol. In each of the three experiments, 9 – 34 cells were analyzed for nuclear translocation. (n= 3; $p < 0.05$ ANOVA; Bonferroni Post hoc test $*p < 0.05$ TSA vs. Control).

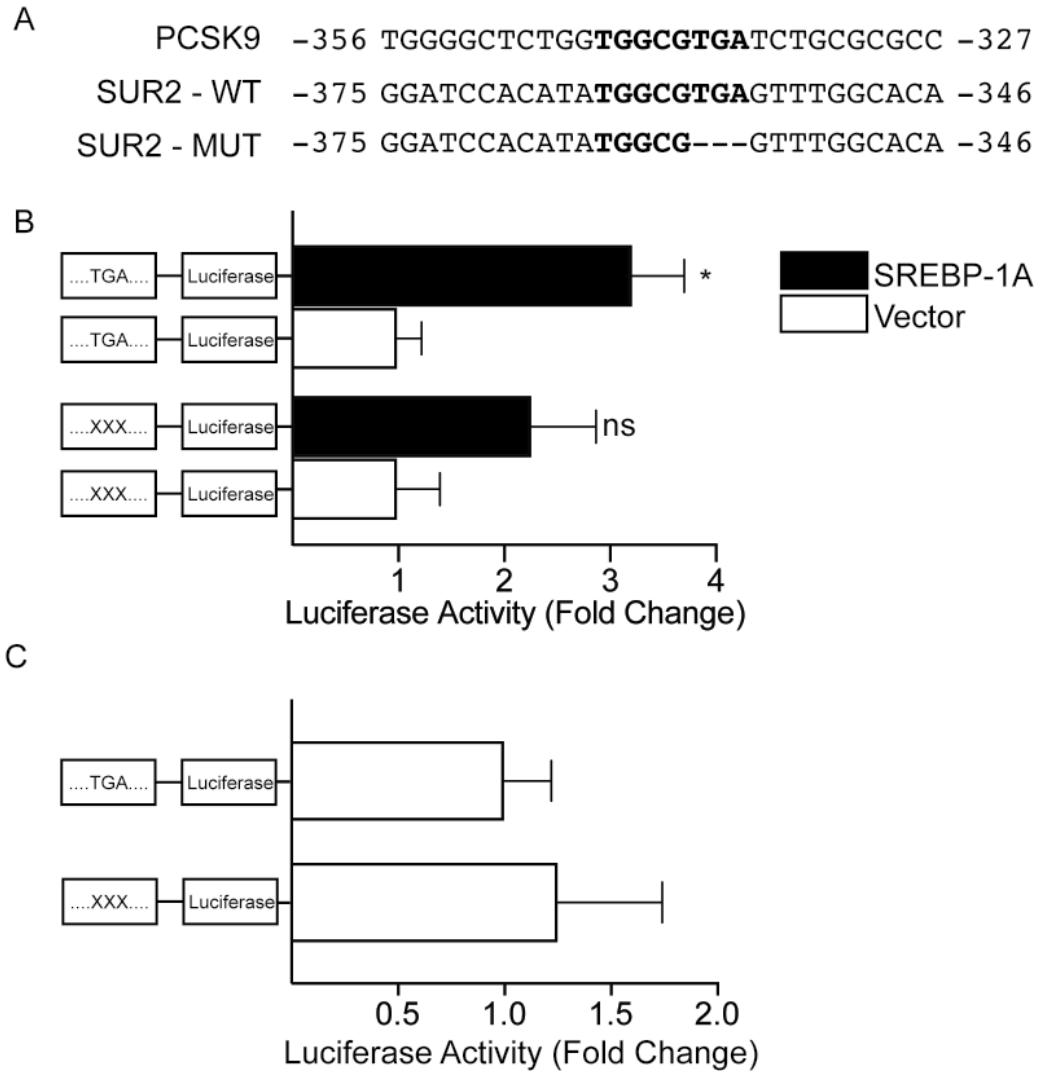


Figure 7: SREBP-1a can activate the SUR2 promoter. (A) 470 bp of the non-coding sequence immediately upstream of the SUR2 transcription start site were cloned into a *Gaussia* luciferase reporter vector. Analysis of this sequence revealed a putative SREBP binding site in the antisense strand (position -356 – -365 relative to the transcription start site) similar to that found in the PCSK9 gene (104). A mutant SUR2 sequence was constructed by deleting three nucleotides shown to be involved in SREBP binding (154). (B) Relative luciferase activity measured in COS-m6 cells transfected with luciferase reporter constructs. Co-transfection with SREBP-1a induced a significant increase in relative luciferase activity compared to co-transfection with vector control. Luciferase activity is not significantly increased when the putative SREBP binding site is mutated, while basal promoter activity (C) was significantly changed not by the mutation. (n=5-10, * $p < 0.05$ Student's T-test.)

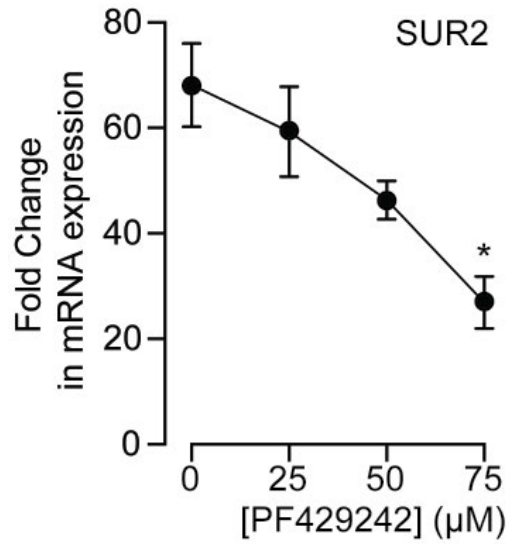


Figure 8: The SREBP inhibitor PF429242 the effect of TSA on SUR2 expression.
Relative mRNA expression in HL-1 cells treated with both TSA and different concentrations of the reversible competitive inhibitor of SREBP maturation, PF429242. Cells were treated with the inhibitor for the final 48 hours of the 72 hour treatment. (n=4-6; $p < 0.05$ ANOVA; Bonferroni Post hoc test * $p < 0.05$ TSA vs TSA+PF429242 75μM.)

ACKNOWLEDGEMENTS /AUTHOR CONTRIBUTIONS/ DISCLOSURE

Disclosures

None

Author Contributions

RG designed and performed experiments, analyzed the data, drafted and edited the manuscript. NF performed experiments and edited the manuscript. JFS maintained HL-1 cells. MCH assisted in data analysis and edited manuscript. TPF designed experiments, analyzed data, and edited the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

This work was supported by funding from the Department of Defense (Early Career Scientist Award to TPF).

Disclaimer: The opinions and assertions expressed herein are those of the author(s) and do not necessarily reflect the official policy or position of the Uniformed Services University or the Department of Defense.

CHAPTER 3: Characterization of K_{ATP} in human iPSC - derived cardiomyocytes

Robert M. Geiger, Naheed Fatima, Michael Klein*, Guzal Khayrullina, Darya Karabasheva, Robert Goldstein*, Mark C. Haigney* and Thomas P. Flagg

Department of Anatomy, Physiology, and Genetics, Uniformed Services University for the Health Sciences, Bethesda, MD, 20814

*Department of Medicine, Uniformed Services University for the Health Sciences, Bethesda, MD, 20814

To whom correspondence should be addressed:

Thomas P. Flagg, PhD

Department of Anatomy, Physiology, and Genetics

Uniformed Services University for the Health Sciences

4301 Jones Bridge Road, Rm. C-2114

Bethesda, MD, 20814

Ph: (301) 295-3509

Fax: (301) 295-3566

e-mail: Thomas.Flagg@usuhs.edu

Running title: hiPSC-CM, K_{ATP} , SUR1, SUR

ABSTRACT

The ATP sensitive potassium channel (K_{ATP}) is a unique ion channel as it links cellular metabolism to channel function. It is a hetero octameric structure composed of four SURx subunits and four Kir6.x subunits. Previously, we demonstrated that cellular cholesterol levels regulate SUR2 mRNA expression in HL-1 cardiomyocytes. Recently, hiPSC derived cardiomyocytes (hiPSC-CM) have become a popular model as it is more comparable to human cardiac physiology. Several ion channels have been characterized in this model, however there is little evidence showing functional K_{ATP} channels. Therefore, we examined hiPSC-CM for the presence of K_{ATP} . We show Kir6.2 protein expression throughout differentiation, while the SURx subunits are expressed later in the time course. Functional studies revealed that P1075, a SUR2 specific K_{ATP} channel activator, can shorten the field potential (FP) and action potential duration (APD). However, we observe only slight FPD shortening at high concentrations of diazoxide, suggesting that SUR2 is the predominant isoform expressed in hiPSC-CM. Finally, we present preliminary evidence that K_{ATP} activation can reduce the amplitude of calcium transients suggesting that the channel could have a cardioprotective role in these cells. Taken together, these studies reveal the presence of functional SUR2 specific K_{ATP} channels in hiPSC-CM.

INTRODUCTION

ATP sensitive potassium (K_{ATP}) channels were first described by Noma in rabbit and guinea pig cardiomyocytes(184). It is now known that K_{ATP} channels are found in a variety of cells, where they respond to intracellular nucleotide levels to modulate cell activity(63). When ATP concentration is sufficient in the cell, K_{ATP} channels are inhibited. A fall in [ATP], coupled with a rise in [ADP], leads to channel activation that reduces cell excitability and limits Ca^{2+} entry into the cell through voltage dependent Ca^{2+} channels.

In the heart, activation of K_{ATP} channels has been implicated as the cause of contractile failure during ischemia and as an effector of ischemic preconditioning(235). It is estimated that activation of just 1% of the K_{ATP} channels in a rat ventricular cardiomyocyte is sufficient to shorten the action potential duration (APD) by 50%(179), limiting calcium entry and contraction(138, 179, 183, 223). By reducing Ca^{2+} entry and limiting contraction, K_{ATP} activation helps preserve high energy phosphate substrates like ATP and creatine phosphate leading to less cell death(33, 162). Furthermore, channel activation reduced the number of detrimental effects due to calcium overload like arrhythmia, contractile dysfunction and cell death(7, 9, 119, 120).

At the molecular level, it is known that K_{ATP} channels are comprised of two distinct subunits assembled in a hetero-octomeric protein complex. Four inward rectifier (Kir6.x; *Kcnj11* or *Kcnj8*) pore-forming subunits combine with four sulfonylurea receptor subunits (SURx; *Abcc8* or *Abcc9*) to form a single functional channel(30, 145, 240). Additional molecular diversity is achieved by alternative splicing of the *Abcc9* gene that gives rise to two major isoforms, SUR2A and SUR2B. Every subunit combination can form a functional channel, however the detailed functional properties of each combination are unique. For example, the SUR subunit determines the efficacy of

potassium channel opening drugs like diazoxide (SUR1>SUR2) and pinacidil (SUR2>SUR1)(99).

Studies to elucidate the molecular composition of K_{ATP} in vivo, its regulation, and its effect on cardiac function have been mostly performed in rodent models. Mouse models in particular provide a significant advantage in the ability to perform genetic modification to study K_{ATP} structure and function. Using this approach, we were able to determine that the molecular composition of K_{ATP} is chamber specific in the mouse heart, with SUR1 and Kir6.2 making up the channel in atrial cardiomyocytes, and with SUR2A and Kir6.2 isoforms making up the channel in the ventricle(30, 64). Interestingly, however, this chamber specific distribution was not apparent in a small cohort of human samples, suggesting that there may be differences between K_{ATP} structure and function in mouse and humans.

While there are many similarities, there are also some key differences in the function of human and mouse heart. The typical heart rate in a mouse is as much as ten times faster than in a human. To accommodate these fast heart rates, the action potential is markedly shorter in mouse cardiomyocytes than in human cells with a much less prominent phase 2 or plateau phase. Additionally, a different cohort of potassium currents contributes to cardiomyocyte repolarization(69). Therefore, to critically examine the structure and function of the K_{ATP} channels in the human heart, new model systems are needed.

Inducible pluripotent stems cell derived cardiomyocytes (hiPSC-CM) have emerged as a potential model to study cardiac function(126, 274). Unlike mouse cardiomyocytes, hiPSC-CM exhibit a long action potential with a prominent plateau phase. Several cardiac disease phenotypes, including long QT syndrome and catecholaminergic polymorphic ventricular tachycardia (CPVT), have been recapitulated in hiPSC-CM (55, 69, 111). hiPSC-CM are also amenable to rapid functional testing. We

recently showed that hydroxychloroquine and azithromycin cause a proarrhythmic phenotype in hiPSC-CM(72), similar to what has been observed in patients treated with these compounds during the recent COVID-19 pandemic(210).

Previous studies have demonstrated that hiPSC-CM express many of the same ion channels found in the heart(94), however it is not clear whether hiPSC-CM express functional KATP channels and what their molecular composition might be.

In this study, we used two different hiPSC-CM models—commercially-available iCells² (Cellular Dynamics, Inc) and cardiomyocytes engineered from the ND2.0 hiPSC line using a small molecule-based approach—to examine the structure and function of KATP. We show that hiPSC-CM express the Kir6.2, SUR1 and SUR2 subunits and use multielectrode array plates (MEA) to examine the field potential in response to the channel activators diazoxide and P1075. Taken together, our results suggest that hiPSC-CM express functional K_{ATP} channels principally comprised of SUR2 and Kir6.2 subunits.

MATERIALS AND METHODS

Cell lines and Culture Methods

Experiments were performed in two different types of hiPSC-CMs as indicated in the text. In some experiments we used icells. In others, we used cardiomyocytes derived from hiPSC-ND 2.0 were obtained from NIH. Cells were passaged using TrypLE Express (Gibco Ref# 12605-010) and E8 (Gibco Ref# A15169-01)+Rock inhibitor (TOCRIS Ref# 1254). ND 2.0 cells were plated at 150,000 cells per well and grown until 90% confluency in Essential 8 (E8) media. Cells were differentiated using a method adapted from Lin, et al. (147). Briefly, on Day 0 hiPSCs were treated with media comprised of (DMEM/F12) plus 10mL/L Fatty acids, 14g/L sodium selenate, 640mg/L Ascorbic acid and holotransferrin, (cardiomyocyte differentiation basal media (CDBM)). Cells were exposed to a GSK3 inhibitor CHIR(5uM) in CDBM for the first 24 hrs for mesenchymal induction, followed by recovery in CDBM+Heparin (3ug/mL) for 1 day. Cells were then treated with the Wnt inhibitor IWP2 (2.5uM) + heparin (3ug/mL) in CDBM for 72 hours followed by 3 days of recovery in CDBM +Heparin (3ug/mL), then 2 days CDBM alone. Selection was performed RPMi - glucose + D-lactate for 4 days, cells were maintained in CDBM after selection.

Western Blot

To obtain protein from western blot analysis, cells were washed with cold PBS and lysed in RIPA buffer with PMSF and Protease inhibitors. Whole cell lysates were sonicated on high power for 1 min and then centrifuged at 10,000 RPM for 5 min at 4° c. Supernatant and pellet were separated. Western blots were performed with lysates normalized to 35-50 ug per replicate. Samples were transferred to PVDF using the semi dry Biorad Turbo iBlot and blocked in 5 % milk in TBS + 0.1% Tween (TBST). Blots were incubated with primary antibody overnight at 4° c, washed with TBST and incubated with

secondary antibody (1:500) for 1 hr. Blots were developed using the super signal West Dura (Thermo Fisher, Cat# 34076). Bands were quantitated using image J.

hiPSC-CM Dissociation and Plating

hiPSC-CM were washed with PBS and dissociated using the stem cell technologies dissociation media for 5-8 min at 37°C. Media was inactivated with CDBM+ 10% FBS and rock inhibitor. Cells were then centrifuged at 500 RPM followed by resuspension in CDBM+ 10% FBS and rock inhibitor, passed through a 70µm filter. Cells were plated at 100,000 cells per well in a 6-well plate at 0.1 mL volume. Cells were plated directly on the coverslip and allowed to adhere for 10 minutes before adding additional media.

Immunofluorescence

hiPSCs-CM on coverslips were washed with PBS, fixed with 4% paraformaldehyde for 10 min and quenched with 0.15% glycine. Cells were permeabilized and blocked with 0.1% triton x-100, 10% FBS in PBS for 1 hr followed by incubation with primary antibody (conc stated in results) in PBS+ 0.1% triton-x 100 at 4 deg. c overnight. Cells were washed and incubated with secondary antibody (1:500) in PBS + 0.1% triton x100 + DAPI (1:10,000) for 1 hr. Coverslips were mounted with Vectashield and sealed with nail polish.

Flow cytometry

In order to determine the number of troponin-t positive cells, hiPSC-CM were washed with PBS and dissociated using the stem cell technologies dissociation media for 5-8 min at 37 c. Media was inactivated with CDBM+ 10% FBS and rock inhibitor. Cells were then centrifuged at 500 RPM followed by resuspension in CDBM+ 10% FBS and rock inhibitor and passed through a 70µm filter. Cells were fixed and stained with

the Biolegend flow cytometry staining kit, analysis was performed on an Accuri C6 flow cytometer (BD).

Analysis of K_{ATP} using multielectrode arrays

We examined K_{ATP} in hiPSC-CMs using multi electrode arrays (MEAs) to determine the change in field potential (FP) after K_{ATP} activation. Field potential is the change in voltage that occurs when an action potential is conducted across a cell monolayer. This metric is analogous to both an electrocardiogram (ECG) and an action potential (AP). It contains a sodium spike similar to both a QRS complex and phase 0 of an action potential, as well as a T wave similar to the repolarization wave in an ECG and phase 3 of an AP. The time between the field potential sodium spike and the peak of the T-wave is analogous to the action potential duration.

Measurement of the local extracellular action potential (LEAP) was used to assess the APD. LEAP uses property protocols to induce strong coupling between a group of cells and an electrode allowing measurement of AP signals arising from hiPSC-CM (89). These signals resemble the cellular AP. APD was calculated from the peak of the AP signal to 30%, 50%, 90% repolarization.

FPD and LEAP was measured using the Maestro MEA reader (Axion). A 24 well plate was coated with 5% fibronectin in PBS for 1 Hr. ICells² were plated in a 24 well maestro plate at 70,000 cells per well according to the manufacturer's protocol. Cells were allowed to attach 7 days prior to drug exposure and measurement in the maestro. Field potential and LEAP were measured 10-15 minutes after drug addition. Drugs were diluted in DMEM+4.5g/L sodium pyruvate (Sigma) DMSO was used for control wells. ICells were paced by field stimulation (800uV, 20mA, 200us) at 1 Hz.

Data Analysis

Data was analyzed using GraphPad Prism software to determine significance between multiple treatments, we used a one-way ordinary ANOVA with a Bonferroni post hoc test. A students t-test was used to determine statistical significance between two groups. Unless otherwise stated, standard error of the mean is shown. The FPD and LEAP was analyzed with maestro or custom software in python.

RESULTS

SUR1, SUR2 and Kir6.2 are expressed in hiPSC derived cardiomyocytes

Previous results have shown that the SURx subunit is the rate limiting step in K_{ATP} biogenesis(201). Therefore, we examined the SUR1, SUR2 and Kir6.2 subunits through the differentiation process using western blot. Protein lysates were harvested at time points after treatments during the differentiation (**Figure 9A**). Western blots (**Figure 9B**) demonstrated an increase in SUR1 and SUR2 expression after IWP2 treatment. However, Kir6.2 is observed throughout the time course, suggesting that expression of the SURx subunits could be the rate limiting step in K_{ATP} ion channel production. Interestingly, we observe reduced amounts of SUR1 after lactate treatment, suggesting that there is more SUR2 than SUR1 in mature hiPSC derived cardiomyocytes. Observations were verified by quantitation of band intensity (**Figure 9C**). In order to ensure cells were becoming cardiomyocyte-like we examined troponin T, a protein involved muscle contraction and a commonly used cell marker specific for cardiomyocytes. Expression of troponin T correlates with expression of the SURx subunits (**Figure 9B**) suggesting that SURx is produced in differentiated cells. Finally, we show that our differentiations achieve approximately 90% troponin T positive cells (**Figure 9C**).

Subcellular Localization of K_{ATP} Subunits

We next sought to determine the subcellular localization of K_{ATP} channel subunits using immunostaining. Images from confocal microscopy show cell fluorescence when probed with SUR2, SUR1 and Kir6.2 antibodies (**Figure 10**). We observe localization of SUR1 to the cytoplasm and SUR2 to the plasma membrane (**Figure 10A**). This suggests that SUR1 is retained from translocating to the plasma membrane, possibly in the ER. However, in later replicates, it is difficult to localize SUR1 and SUR2 (**Figure**

10B). Therefore, we cannot determine where the subunits are localizing from the micrographs. In order to further examine subunit localization, we assessed K_{ATP} function in hiPSC-CM using multielectrode arrays (MEAs).

P1075 activates the K_{ATP} channel more than Diazoxide

Given the apparent cell surface localization of SUR2 and Kir6.2, we first tested the effects of P1075, an SUR2 specific agonist. We observe a dose dependent shortening of the FP with an IC_{50} of $0.207\mu M$ (**Figure 11A**). The K_{ATP} channel inhibitor glibenclamide ($1\mu M$) prevents the reduction in FPD (**Figure 11B**). We also tested the effects of diazoxide on FPD. Unlike P1075, significantly more diazoxide ($300\mu M$) is needed to get any FPD shortening (**Figure 11A**). Taken together, this shows that activation of SUR2 containing K_{ATP} can cause FPD shortening.

We next examined the action potential duration (APD) in response to KCOs. We observe more APD shortening with P1075 ($0.2\mu M$) compared to diazoxide ($300\mu M$) (**Figure 12A**). We also show a reduction of APD 30, 50 and 90 suggesting that the channel is activated throughout the entire APD (**Figure 12B**). Taken together, this data suggests that hiPSC-CM express functional SUR2 containing K_{ATP} channels, and that activation occurs throughout the AP.

DISCUSSION

The data in this study demonstrate that hiPSC-CM express K_{ATP} channel subunits and activation with the K_{ATP} selective potassium channel openers caused shortening of the field potential duration (FPD) and local extracellular action potential (LEAP) reveal that the channels are functional. Taken together, these results suggest that hiPSC-CM could be an important model system to study K_{ATP} structure and function.

SURx as the rate limiting subunit for K_{ATP} channel expression

Interestingly, we found that the pore-forming Kir6.2 channel subunit is expressed in undifferentiated hiPSC, but differentiation of the cells into cardiomyocytes induced the expression of both SUR1 and SUR2 subunits, coincident with the induction of TnT **(Figure 9)**. Since K_{ATP} channels are obligate heteromultimers, this result suggests that formation of functional K_{ATP} is limited by the expression of SURx subunits in hiPSC. This is in line with previous observation in guinea pig showing SUR2a is the rate limiting subunit(201).

hiPSC-CM express predominantly SUR2 K_{ATP} channels

We previously showed that the molecular composition of sarcolemmal K_{ATP} is chamber-specific in the mouse heart(64). SUR1 is the predominant isoform making up the atrial channel, while SUR2 is the major isoform in ventricular myocytes. In the present study, we found that high doses of diazoxide, the SUR1 specific K_{ATP} channel activator, only modestly reduced the FPD and APD. Conversely, the SUR2 specific channel opener, P1075, dramatically reduced FPD and APD with an apparent affinity of ($IC_{50} = 0.207\mu M$). This result suggests that the sarcolemmal K_{ATP} channels hiPSC-CMs are primarily composed of the SUR2 and Kir6.2 subunits. It has been suggested that the cells generated by most hiPSC-CM differentiation protocols are ventricular-like(16), and

the present finding would be consistent with this conclusion. It should be pointed out, that data from human hearts suggest that SUR1 may also be an important component of channels in ventricular cardiomyocytes(59). hiPSC-CM are derived from human cells therefore, this may be why we observe some shortening of the FPD with diazoxide even though most hiPSC-CM protocols generate cells that are ventricular-like.

We also observe that the expression of both SUR1 and SUR2 are induced during differentiation of hiPSC to form hiPSC-CMs. It is surprising that both SUR1 and SUR2 subunits are expressed, but the pharmacological profile suggests that functional channels principally contain the SUR2 subunit (**Figure 11**). It is unclear why this should be the case, since previous studies have shown that all channel subunit combinations can occur so long as the subunits are expressed(25, 38).

Examination of SUR2A and SUR2B in hiPSC-CM

SUR2 is expressed as two splice variant SUR2A and SUR2B(28, 66, 110, 224) SUR2A is the predominant splice variant in ventricular cardiomyocytes(5, 31, 64, 149). We did not directly examine the SUR2A or SUR2B splice variants in hiPSC-CM; however, the results suggest that SUR2A is likely to be the predominant splice variant expressed in the cells. Diazoxide has been shown to activate both SUR2B and SUR1 K_{ATP} channels, while P1075/pinacidil activates both SUR2A and SUR2B(6, 157, 170, 253). Therefore, if SUR2B were a major component of the sarcolemmal K_{ATP} channel in hiPSC-CMs, we would expect both diazoxide and P1075 to shorten the FPD and APD with a similar affinity. Instead, we observed that P1075 shortens the FPD with high affinity, while diazoxide only affects the FPD at significantly higher concentrations. Further analysis needs to be performed in order to confirm expression level of the different splice variants in hiPSC-CM, however the present results indicate that SUR2A is the predominant SUR2 splice variant in hiPSC-CM.

hiPSC-CM as a model to study disease causing mutations

Cantu syndrome is a rare disease caused by mutations in either the SUR2 or Kir6.1(18, 87). Cantu syndrome is characterized by hypertrichosis, craniofacial dysmorphism, osteochondrodysplasia, patent ductus arteriosus, cardiomegaly, pericardial effusion, and lymphoedema(183). Analysis of the direct effects of Cantu-associated mutations on cardiac function in mouse models of the disease are complicated by potential effects on the vascular and lymphatic systems that can indirectly affect cardiac function(41, 127). The results of this study show that hiPSC-CM express functional K_{ATP} , raising the possibility that iPSCs could be obtained from patients with Cantú syndrome to study the direct effect of mutations on cardiac function.

Conclusion

In summary, our results demonstrate that hiPSC-CM express functional K_{ATP} channels. Functional channels are principally comprised of SUR2A subunits, similar to what is observed in adult ventricular cardiomyocytes. Therefore, hiPSC-CM represent a novel model system to study the structure and function of K_{ATP} during normal development, in response to physiological and pathological metabolic stress and in K_{ATP} associated diseases like Cantú syndrome.

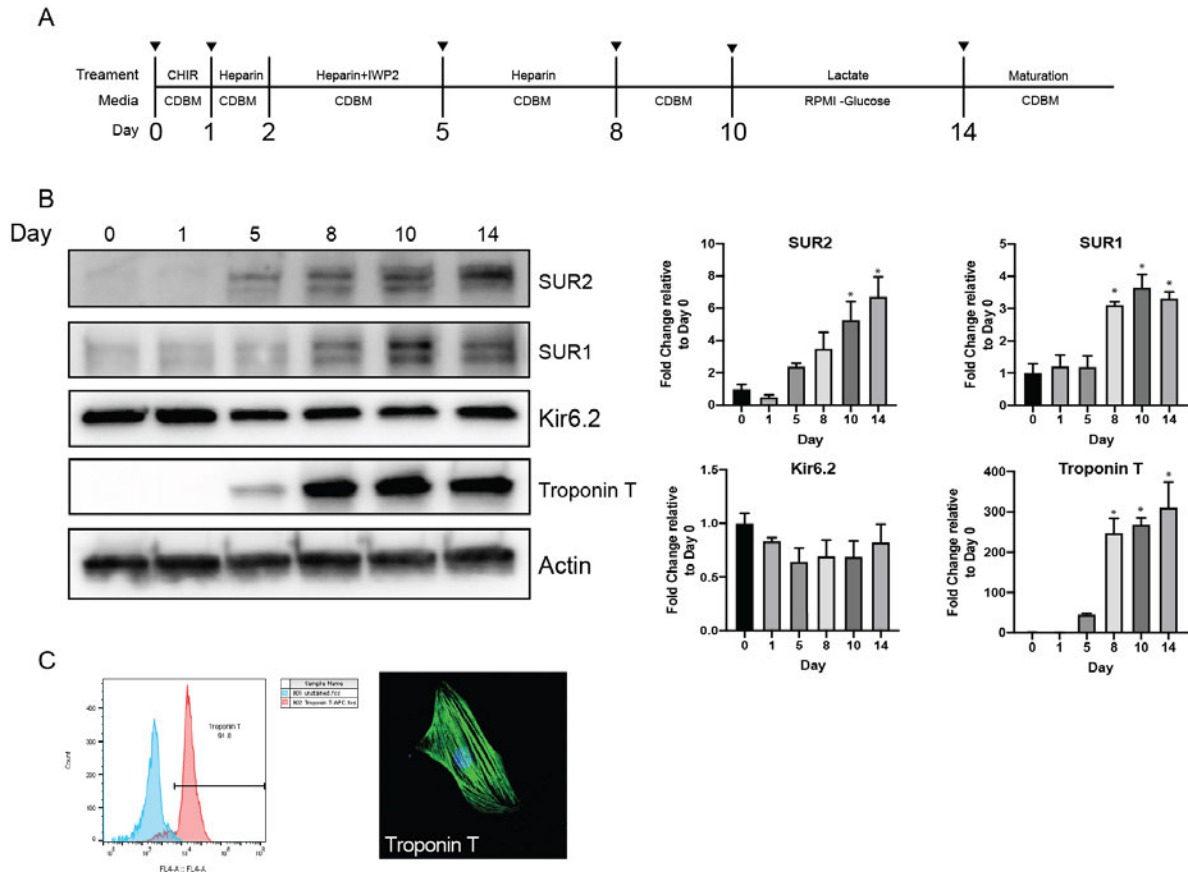
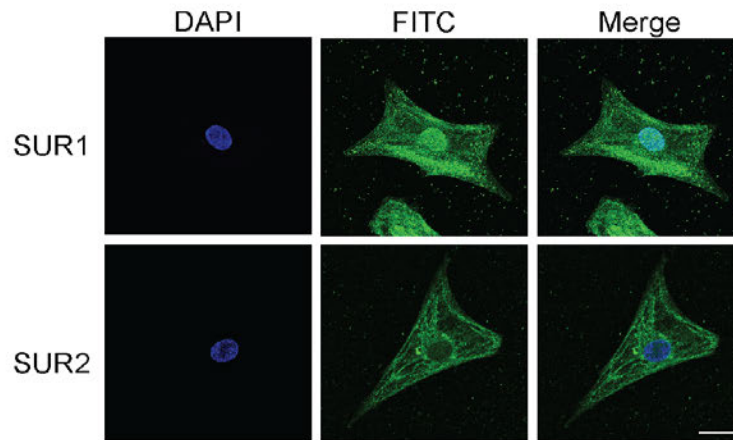


Figure 9: Induction of the SURx subunits correlates with Troponin T during Differentiation. (A) Shown are representative western blot results demonstrating the expression of KATP subunits (SUR1, SUR2, Kir6.2) and a marker of cardiomyocyte differentiation, troponin T(TnT). (B) Quantitation of results from blots as in A reveals a marked induction of SUR1 and SUR2 protein expression corresponding with cardiomyocyte differentiation and the appearance of TnT. (C) Troponin T levels were assessed with flow cytometry, cells stained anti – troponin T shown in the red histogram compared to unstained cells shown in blue. Representative confocal micrograph of a hiPSC-CM stained with anti – TnT (*, $p < 0.05$; ANOVA, $n = 3$)

A



B

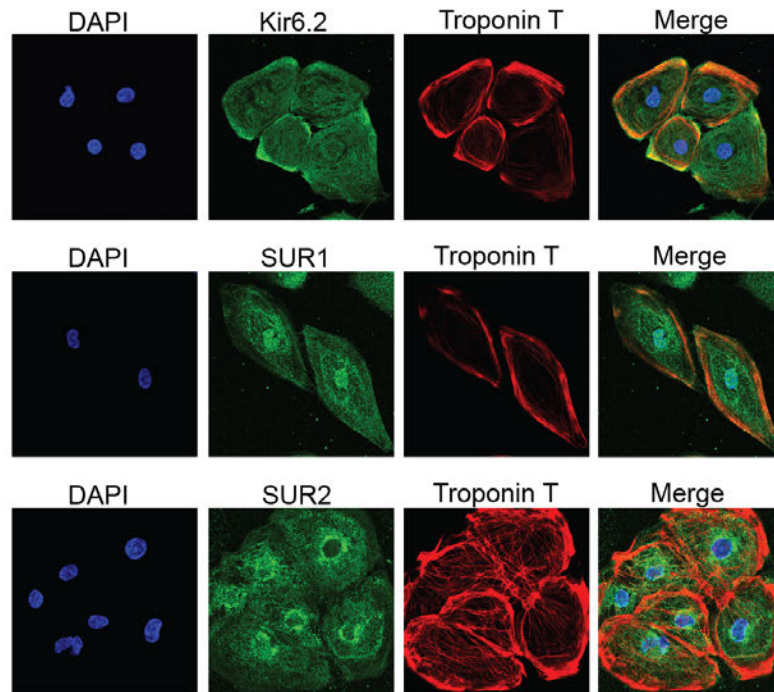


Figure 10: Confocal images suggesting the SUR1 subunits may be retained in the ER. (A) Representative images of hiPSC-CM showing SUR1 may be retained in the ER and SUR2 in the plasma membrane. **(B)** Representative images showing subcellular localization of K_{ATP} channel proteins (SUR1, SUR2, Kir6.2) throughout the cell in hiPSC-CM. Given the inconsistent results, further experiments are needed in order to localize the K_{ATP} channel proteins.

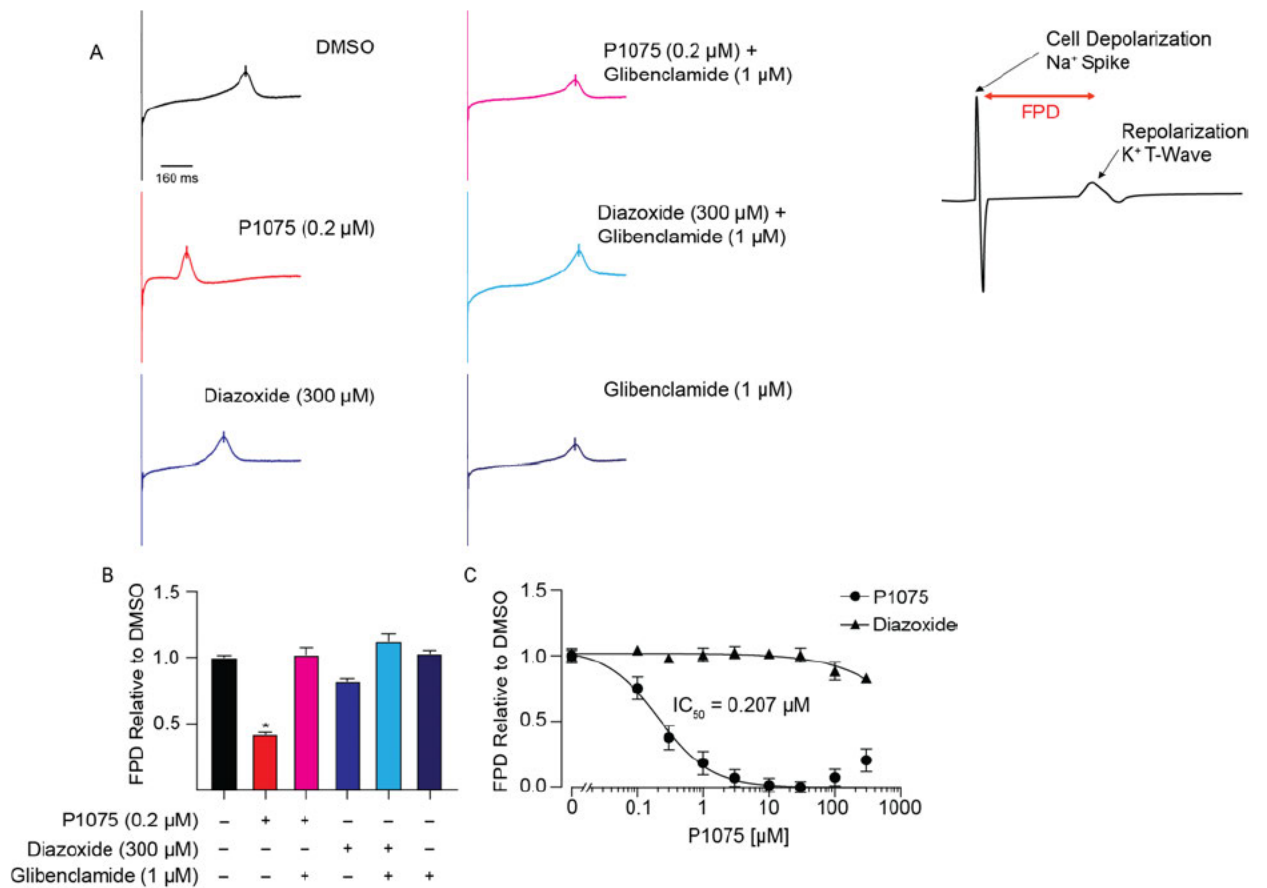
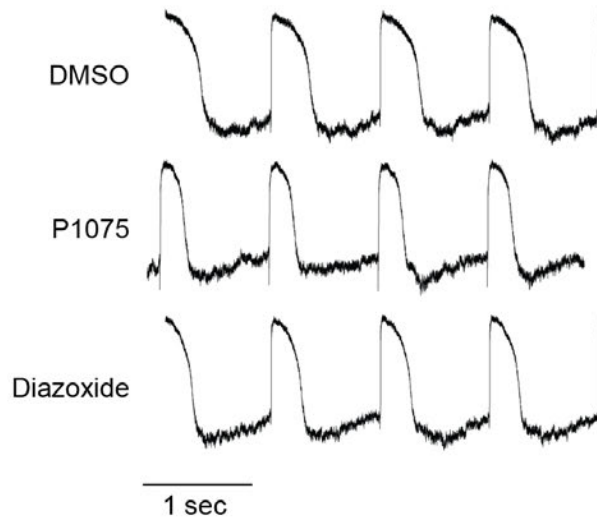


Figure 11: FPD is shortened by the SUR2-selective agonist P1075 (A) (A)

Representative field potential recordings that have had part of the sodium spike removed to better view the T-waves and **(B)** summary analysis of FPD in hiPSC-CM (iCells²) monolayers treated with SUR2-selective KATP agonist, P1075. The FPD was measured from the “Na⁺ spike” to the peak of the “T wave”. P1075 induced marked shortening of the FPD, that was inhibited by glibenclamide (1 μ M). **(C)** While P1075 (SUR2 agonist) induces a marked concentration – dependant (IC₅₀ = 0.207 μ M) shortening of the FPD, the SUR1-selective KATP agonist had little or no effect of the FPD. (*, $p < 0.05$; ANOVA, $n = 2-5$). **(D)** Schematic showing the time between the cell depolarization spike and the repolarization T – Wave is the field potential duration (FPD).

A



B

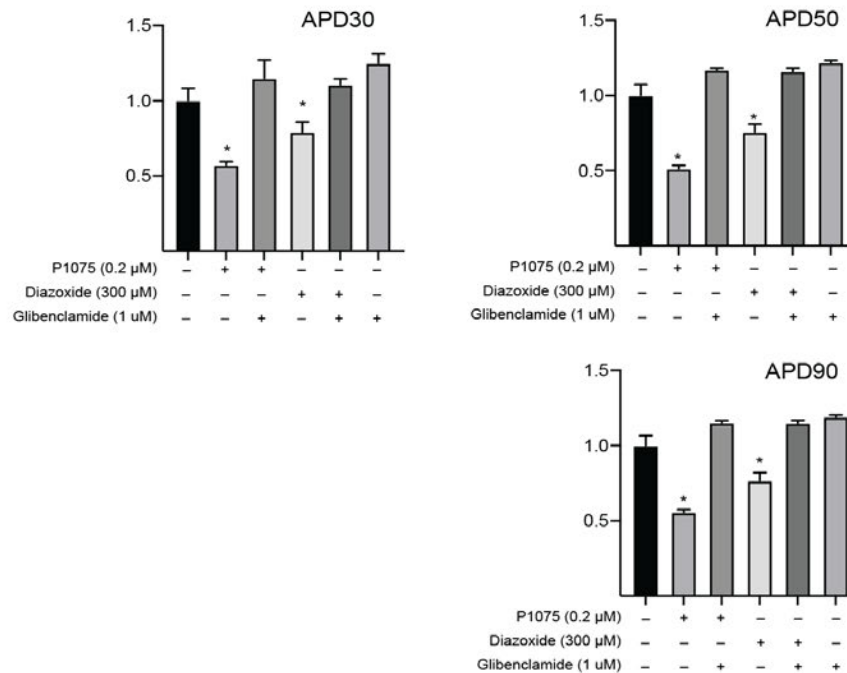


Figure 12: APD90 is Reduced by the SUR2-Selective K⁺ Agonist, P1075. (A) Shown are local extracellular action potentials (LEAP) recordings from hiPSC-CM monolayers paced at 1 Hz and treated with the indicated K_{ATP} agonists. The amplitude of the LEAPs were normalized to the maximum in each trace. (B). Summary results demonstrating that P1075 causes a significant APD30, 50, 90 shortening in a concentration dependent fashion, while diazoxide has little effect on APD90. (*, p < 0.05; ANOVA, n = 2-5)

ACKNOWLEDGEMENTS /AUTHOR CONTRIBUTIONS/ DISCLOSURE

Disclosures

None

Acknowledgements

This work was supported by funding from the Department of Defense (Early Career Scientist Award to TPF).

Disclaimer: The opinions and assertions expressed herein are those of the author(s) and do not necessarily reflect the official policy or position of the Uniformed Services University or the Department of Defense.

CHAPTER 4: Rapid Assessment of Proarrhythmic Potential using Human iPSC-Derived Cardiomyocytes

Robert M. Geiger[†], Michael G. Klein PhD^{*†}, Naheed Fatima, Robert E. Goldstein, MD^{*},
Mori J. Krantz, MD[‡], Mark C. Haigney, MD^{*}, and Thomas P. Flagg, PhD[‡]

Department of Anatomy, Physiology & Genetics and ^{*}Department of Medicine,
Uniformed Services University, Bethesda, MD, 20814.

[‡]Denver Health Medical Center, Denver CO, 80204-4507

[†]These authors contributed equally to this work

Correspondence[‡]:

Thomas P. Flagg, PhD
Department of Anatomy, Physiology & Genetics
Uniformed Services University
Email: thomas.flagg@usuhs.edu
Phone: (301)295-3509

Word Count: 793

ABSTRACT

Rapid cardiac safety testing is required as the misuse or repurposing of existing drugs increases in response to emergent health threats. This is clearly evident in the ongoing epidemics of opioid abuse (loperamide) and COVID-19 (hydroxychloroquine and azithromycin). Here we demonstrate rapid evaluation of the proarrhythmic potential of these compounds using a microelectrode array system to monitor human iPSC-derived cardiomyocyte monolayer excitability. These results demonstrate the feasibility and potential of this experimental approach to identify cardiovascular risks of existing drugs being used in unintended ways, drugs being used in novel combinations and therapies in development.

INTRODUCTION

Public health threats like the ongoing epidemics of opioid abuse and COVID-19 can result in restriction of existing drugs or their repurposing in new and potentially dangerous ways. For example, ingestion of high doses of loperamide, an over-the-counter mu-opioid agonist, has increased as opioid-dependent patients seek inexpensive, widely available alternatives in response to stringent prescription drug monitoring programs. Similarly, the use of hydroxychloroquine alone or in combination with azithromycin has been touted as a treatment for SARS-CoV-2 infection, despite an apparent lack of therapeutic benefit (73). Because these drugs are not being used as intended (loperamide) or used in combinations that have not been well studied (hydroxychloroquine and azithromycin), there is little cardiac safety data on these drug and dosing regimens. Importantly, the ability to perform randomized control trials requires time that may be in short supply during an epidemic. Developing approaches to rapidly assess cardiac rhythm stability in a controlled manner are needed to provide timely guidance to ensure patient safety.

To address this need, we examined electrical activity in human-induced pluripotent stem cell (iPSC) derived cardiomyocyte monolayers using the Axion Maestro Microelectrode Array (MEA) system to rapidly assess potential adverse drug effects on cardiac excitability. All studies were performed on commercially available iPSC-derived cardiomyocytes (iCell²; Cellular Dynamics) and did not require institutional review board approval. Cardiomyocytes were thawed and plated as monolayers on fibronectin-coated MEA plates (24 well; 70,000 cells/ well) and cultured for 5 to 12 days before recording. The extracellular field potential duration and amplitude of the early spike (P_{Max}) reflect the mean action potential duration and sodium (Na) conductance, respectively,

analogous to the QT interval and QRS complex assessed in a surface electrocardiogram. We also measured the local extracellular action potential (LEAP) that resembles the monophasic action potential. In all experiments, cells were maintained at 37°C and paced by field stimulation at 1 Hz.

During the ongoing opioid epidemic, a growing number of individuals have presented to emergency departments with QT prolongation and Torsade de pointes associated with ingesting supratherapeutic doses of loperamide. Loperamide, marketed primarily in the United States as Imodium (Johnson & Johnson, New Brunswick, New Jersey), is a peripherally acting mu-opioid agonist used to treat diarrhea. We previously demonstrated that loperamide is a potent inhibitor of the human cardiac ether a` go-go–related gene (hERG), encoding the rapid delayed-rectifier potassium-current (I_{Kr}), which increases the QT-interval duration and risk of Torsade de pointes (267).

RESULTS

When applied to iPSC-derived cardiomyocytes (2 to 5 h) (**Figure 13A**), loperamide caused a concentration- dependent increase in the FPD (IC₅₀ = 51.9 nM). Moreover, after prolonged exposure (24 h) (**Figure 13B**), higher doses resulted in the emergence of distinct markers of proarrhythmic electrical activity in LEAP recordings, manifested as AP prolongation (APD₉₀) by >10% (Fridericia correction), early after-depolarizations (EADs) and noncapture by pacing stimuli. Arrhythmia risk markers rarely emerged at the 3-nM concentration, which is near the expected therapeutic serum concentration (< 2 ng/ml), but frequent induction of rhythm instability was observed at supratherapeutic concentrations (300 nM), corroborating mounting cardiac safety concerns for loperamide during the opioid crisis.

In the face of the COVID-19 pandemic, repurposing of hydroxychloroquine, an antimalarial drug, and azithromycin, a macrolide antibiotic, was promoted as a treatment strategy to combat the disease. Like loperamide, hydroxychloroquine is a multichannel inhibitor, particularly of I_{Kr} (22) and would be expected to prolong the QT interval. However, in the setting of a multidrug regimen, concurrent blockade of depolarizing currents may mitigate these effects (117). Azithromycin inhibits I_{Na} and I_{Ca} , with only low affinity blockade of I_{Kr} , whereas long-term exposure can increase I_{Na} (270). Therefore, the net effect of this drug combination on cardiac excitability is difficult to predict but can be rapidly tested using iPSC-derived cardiomyocytes.

Exposure to equal concentrations of both hydroxychloroquine and azithromycin increased FPD in a concentration-dependent manner ($IC_{50} = 0.73$ mM) (**Figure 14A**). Hydroxychloroquine alone prolonged the FPD over a similar range of concentrations, as did azithromycin, but with 4-fold lower sensitivity. Although hydroxychloroquine-dependent block of potassium channels received the most attention, we also observed a decrease in the depolarization spike amplitude by hydroxychloroquine and azithromycin (P_{Max}) (**Figure 14A**), which suggested that both drugs also inhibit depolarizing I_{Na} or I_{Ca} equally. Proarrhythmic activity was also observed in LEAP recordings during prolonged exposure (**Figure 14B**). Exposure to hydroxychloroquine alone caused APD90 prolongation, frequent EADs, and loss of stimulus capture, whereas azithromycin caused a loss of stimulus capture with an increase in the intrinsic beat rate. However, EADs were not observed with azithromycin, either alone or in combination with hydroxychloroquine. These results suggested that this specific drug combination should be restricted to patients undergoing electrocardiographic monitoring.

DISCUSSION

The present study demonstrated the feasibility and usefulness of using iPSC-derived cardiomyocytes to rapidly assess and stratify proarrhythmic risks of drugs that are abused (e.g., loperamide) or applied in uncharacterized combinations (e.g., hydroxy-chloroquine and azithromycin). This novel approach to evaluating pharmacodynamic effects on cardiac rhythm stability has the potential to rapidly identify clinical risks associated with diverse treatment strategies.

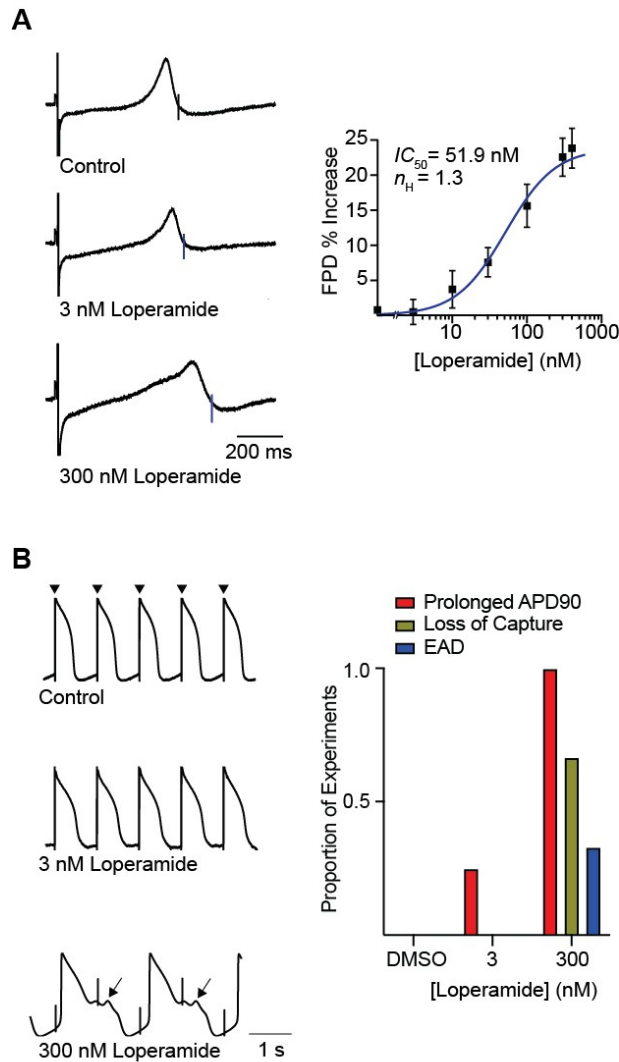


Figure 13: Evaluating proarrhythmic potential of loperamide using human iPSC-derived cardiomyocytes. (A) Representative field potentials recorded from iPSC-derived cardiomyocyte monolayers before and 2-5 hours after loperamide exposure at the concentrations indicated. The field potential duration (FPD) was measured from the initial depolarization spike (analogous to the QRS complex) to the end of the T-wave (as indicated by the vertical line). FPD was unaffected at therapeutic (3 nM) concentrations but markedly increased at supratherapeutic (300 nM) concentrations. Data points in the dose-response curve represent means $\pm$ SEM ($n = 4-7$). Curves represent the least squares fit to the data with a Hill equation (Hill coefficient = 1.3). **(B)** Shown are exemplar Local Extracellular Action Potentials (LEAPs) from the same cells recorded after 18-25 hours exposure to loperamide at the indicated concentrations. At supratherapeutic concentrations, LEAP recordings revealed frequent proarrhythmic electrical activity, including > 10% prolongation of the action potential duration (red), early afterdepolarizations (EADs; arrows, blue bar), and failure of stimulus capture (green). Bar-graph represents the proportion of wells exhibiting the indicated proarrhythmic phenotype ($n = 3-4$).

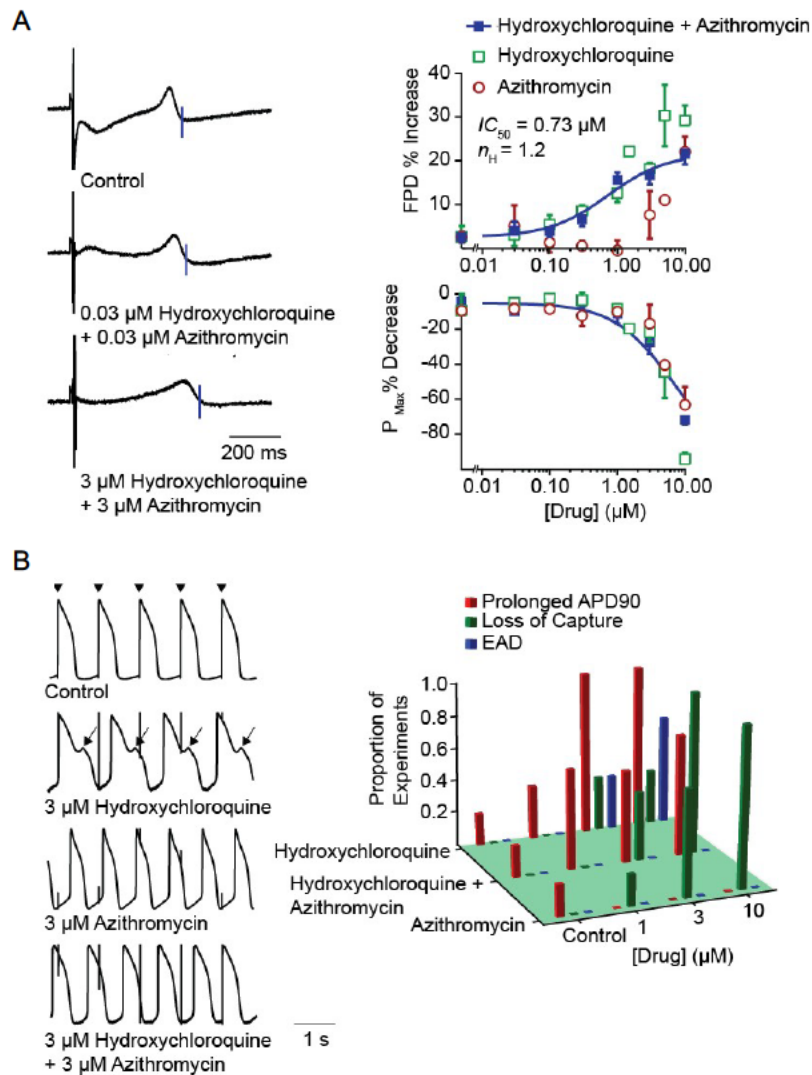


Figure 14: Evaluating proarrhythmic potential of hydroxychloroquine and azithromycin using human iPSC-derived cardiomyocytes.

(A) Representative field potential recordings illustrating FPD prolongation in cardiomyocytes exposed to increasing concentrations of hydroxychloroquine and azithromycin. Dose response curves represent the least squares fit of the data with a Hill equation (Hill coefficient = 1.2) ($n = 4-8$). **(B)** Sample LEAP records from cells treated with either hydroxychloroquine, azithromycin or both in combination. Summary results illustrate the frequency of monolayers exhibiting each identified proarrhythmic activity (EADs, *blue*; APD prolongation, *red*; loss of stimulus capture, *green*) with each of the treatments. APD prolongation and loss of stimulus capture were evident in cells treated with both hydroxychloroquine and azithromycin, however EADs were absent suggesting a complex interplay of the proarrhythmic consequences of the combined therapy ($n = 3-8$). All recordings were analyzed with the cardiac module of the Maestro Pro software or custom software programmed in Python or ID.

COMPETING INTERESTS/AUTHOR CONTRIBUTIONS/ DISCLAIMER

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

RG and MGK designed and performed the experiments, analyzed the data, drafted and edited the manuscript. NF assisted with experimental design. MCH, REG, MJK and TPF drafted and edited the manuscript. All authors have read and approved the final manuscript.

Disclaimer

The opinions and assertions expressed herein are those of the author(s) and do not reflect the official policy or position of the Uniformed Services University or the Department of Defense.

CHAPTER 5: Discussion

MAJOR FINDINGS

In Chapter 2, we demonstrated a novel role for the cholesterol sensitive transcription factor, sterol response element binding protein (SREBP), as a regulator of SUR2 expression in HL-1 cardiomyocytes. In Chapter 3, we showed that hiPSC-CM express functional K_{ATP} channels comprised principally of SUR2 and Kir6.2. Finally, in Chapter 4, we used multielectrode arrays to rapidly assess the cardiotoxicity of drugs being used in novel and unintended ways during an emergent health crisis. Collectively, these observations have implications for our understanding of how K_{ATP} channels are assembled and reveal novel ways to test ion channel function in human cardiomyocytes.

REGULATION OF K_{ATP} CHANNEL BIOGENESIS

The K_{ATP} channel is an obligate hetero octamer composed of 4 SURx and 4 Kir6.x subunit. This requirement is due to the RKR ER retention motif found on the Kir6.2 subunit(275). If SURx is not coexpressed with Kir6.x, the RKR motif will be exposed and the subunit retained in the ER(98, 251, 275). We show that the Kir6.2 subunit is expressed in undifferentiated hiPSC, while expression SURx is observed later in the time course. We hypothesize that expression of the SURx subunit is the rate limiting step in K_{ATP} generation. This is consistent with previous studies suggesting that female guinea pigs generate more functional K_{ATP} channels compared to male pigs as they express more SUR2A(201). Therefore, it would be important to identify the mechanisms regulating SUR2 expression.

Transcriptional regulation of the subunits could also play a role in channel generation. We show that SUR2 expression is regulated by cellular cholesterol levels

through the sterol response element binding protein (SREBP), a transcription factor involved in regulating genes involved in fatty acid and cholesterol biosynthesis(56). While we did not examine the number of K_{ATP} channels generated after cholesterol depletion, we speculate there to be increased K_{ATP} current in these cells as expression of SUR2 is suggested to be the rate limiting step in K_{ATP} generation(201).

SREBP may be involved in subunit expression in other cell types. It would be interesting to examine this mechanism in hiPSC-CMs as it is more comparable model to human physiology. SREBP regulates expression of genes involved in lipid and cholesterol metabolism(97). hiPSC-CM are shown to increasingly rely on oxidative metabolism and fatty acids as they differentiate and mature suggesting involvement of SREBP(70). Curiously, we observe increased SUR2 protein as the cells differentiate (**Figure 9**) suggesting that SREBP could be regulating SUR2 expression in hiPSC-CM.

Since we show the K_{ATP} subunits are already present in hiPSC-CM, we propose using exogenous cholesterol to determine if there is a decrease in subunit expression followed by assessment of the FPD using multielectrode arrays and ultimately K_{ATP} current using patch clamp.

hiPSC-CM AS A MODEL TO STUDY K_{ATP} FUNCTION DURING METABOLIC STRESS

A major consequence of K_{ATP} activation is to reduce cell excitability and calcium entry to protect cells from calcium-induced damage(8, 9, 82, 181). We provide preliminary data suggesting that activation of sarcolemmal K_{ATP} causes a reduction in the amplitude of the calcium transients (**Figure 15**), similar to what is observed in isolated adult cardiomyocytes exposed to P1075 or metabolic stress(235).

Activation of K_{ATP} is shown to be cardioprotective in a mouse model of ischemia reperfusion injury (33, 162). It would be interesting to know if K_{ATP} was cardioprotective

in hiPSC-CM as well. Coverslip hypoxia has previously been suggested as a potential model to simulate ischemia(195). We speculate that using the coverslip model of

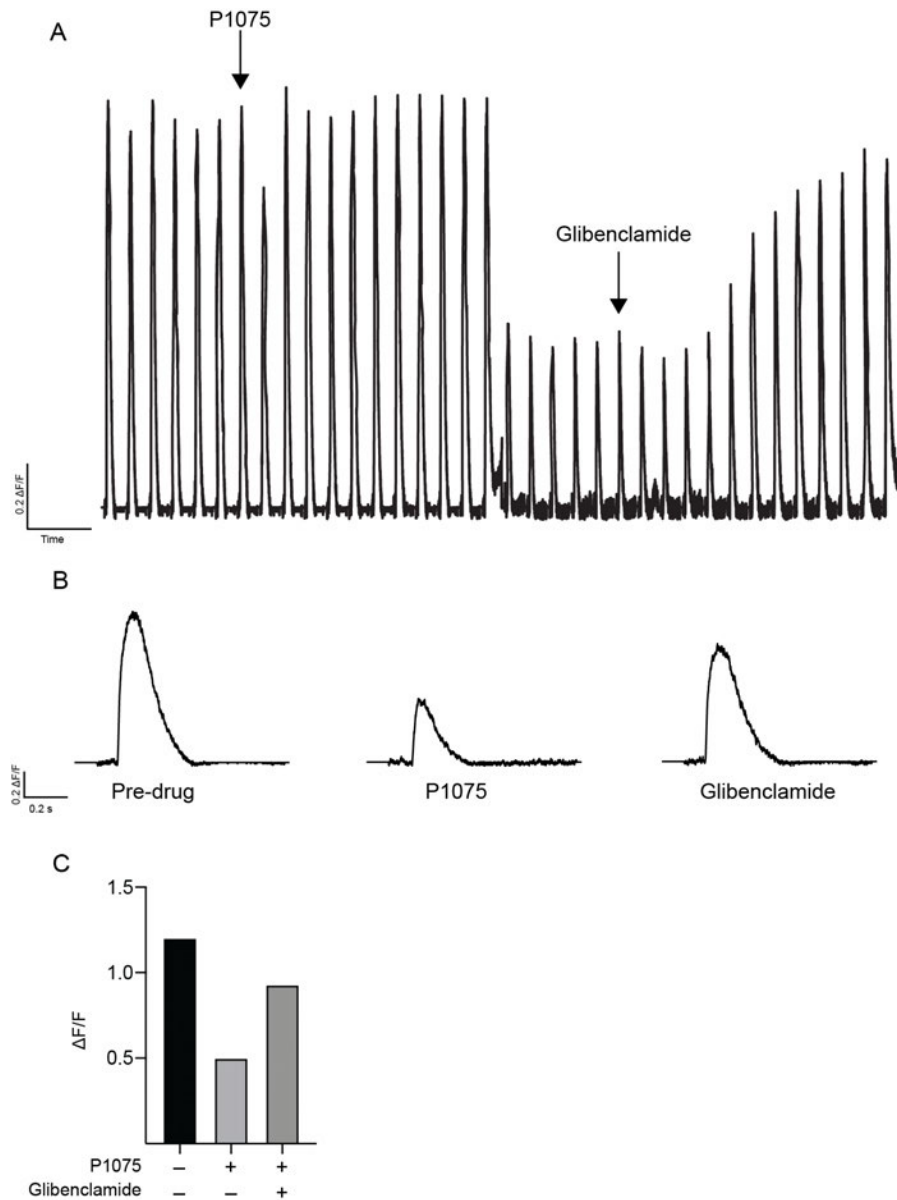


Figure 15: K^+ Activation Reduces Cytoplasmic Ca^{2+} Transient Amplitude in hiPSC-CMs (A) Condensed recording illustrating the effects of K_{ATP} activation by P1075 on the amplitude of the Ca^{2+} transient. Each transient is the average of the three consecutive waveforms. (B) Representative calcium transients averaged from the last four wave forms of drug exposure. (C) Summary data showing the change in fluorescence after cells we exposed to each drug.

hypoxia in hiPSC-CM could be used to probe the K_{ATP} dependent mechanisms of cardioprotection in human cardiomyocytes. Another common method to activate K_{ATP} and examine cardioprotection is through simulated ischemia using the metabolic inhibitors cyanide and 2-deoxy-D-glucose(33). hiPSC-CM could be stressed with simulated ischemia to determine if K_{ATP} inhibition caused a reduction in cardioprotection and more cell death.

Although shortening the APD is demonstrated to be cardioprotective, K_{ATP} activation can also promote reentrant arrhythmias(43). We observed marked shortening of the FPD, however we did not observe evidence of reentry. This is likely because we did not use an arrhythmia inducing stimulus protocol (e.g. burst pacing or premature stimuli) to induce reentry in the monolayer. Going forward, we could use an arrhythmia inducing protocol in addition to increasing the size of the cell monolayer in order to improve our chances of observing an reentrant arrhythmia.

We examined the changes in field potential duration (FPD) in a monolayer of cardiomyocytes. A field potential exhibits similarity to an action potential (AP) and an electrocardiogram (ECG). An AP is the change in membrane potential from a single cell. The FP reflects the change in extracellular voltage near a monolayer of cells. Similarly, an ECG correlates with the change in voltage as an AP is conducted through the heart. The time between the depolarization spike and the T wave of an FP is analogous to the time between phase 0 and phase 4 of the cardiac AP, in the same way that the QT interval of an ECG reflects the AP. At the cellular level, activation of K_{ATP} causes a shortened APD, consistent with our results showing a reduced FPD with K_{ATP} activation **(Figure 11)**.

In an ECG, K_{ATP} activation is observed as ST segment elevation. During a heart attack, K_{ATP} is activated in the ischemic tissue setting up the situation where most of the heart exhibits a normal APD, but the APD of the ischemic area is shorter due to K_{ATP}

activation. This difference is observed as ST segment elevation in an ECG(145, 146). We did not observe any changes in FP elevation. This is because we were measuring the FP at one electrode, not the difference in potential between two electrodes like in an ECG. Furthermore, in our assay the entire monolayer was exposed to K_{ATP} activator, therefore the channel was activated throughout the well not just in a specific area as would be expected in a heart attack.

COMPARISON BETWEEN MATURE ADULT CARDIOMYOCYTES AND hiPSC-CM

We often refer to hiPSC-CMs as “cardiomyocyte-like cells” as there are many differences between these cells and mature adult cardiomyocytes. For example, protocols for making hiPSC-CM generate a mix of cells that exhibit ventricular, atrial and nodal action potentials(147). It has been suggested that most protocols produce ventricular-like cells(151). Since SUR2 is expressed predominantly in the ventricle, this could account for why we observe more FPD shortening with P1075 compared to diazoxide.

Unlike mature adult cardiomyocytes, hiPSC-CMs lack Kir2.x, a potassium current (I_{K1}) critical to setting the resting membrane potential(42, 77). Therefore, the FPD prolongation and proarrhythmic markers that we observe in our hiPSC-CM are likely due to block of other ion channels, possibly hERG. If hiPSC-CM had I_{K1} , we speculate that hydroxychloroquine exposure may cause delayed after depolarizations (DADs), another type of proarrhythmic marker. While some groups have suggested ways to improve maturity and I_{K1} levels, it is generally accepted that hiPSC-CM are not an ideal model to study this channel(58, 77, 255).

It is interesting to speculate why hiPSC-CM may not express Kir2.x. It has previously been shown that contraction plays an important role in cardiac development(277). In hiPSC-CM, pacing after differentiation is shown to help the cells

mature(92, 239). If the cells had Kir2.x they may not be able to spontaneously beat as their resting membrane potential would be set to E_K . Therefore, I_{K1} current may interrupt differentiation.

ASSESSING DRUG INDUCED CARDIOTOXICITY WITH hiPSC-CM YIELDS COMPARABLE RESULTS TO CLINICAL TRIALS

We show that hydroxychloroquine and/or azithromycin caused a prolonged FPD as well as several proarrhythmic markers in hiPSC-CM (**Figure 14A, B**). Consistent with our data, several recent clinical trials report that hydroxychloroquine or azithromycin alone, or in combination, can increase the incidence of QT prolongation in patients with COVID-19 when compared to patients not given the drugs(24, 123, 231). One clinical trial found that 23% of patients developed severe QT prolongation defined as a QT interval > 500 ms(27). Another study showed that using azithromycin and hydroxychloroquine in combination prolonged the QT-interval to a greater extent than using the drugs individually suggesting an additive effect(200). Consistent with this, we show that treating hiPSC-CM to a combination of hydroxychloroquine and azithromycin result in a combination of arrhythmogenic markers found in each of the drugs alone also suggesting an additive effect of the drugs.

We also examined the “sodium spike” or the P_{max} of the field potential. The sodium spike reflects the upstroke of the FP and therefore is a function of the depolarization current in the cell - I_{Na} . Both hydroxychloroquine and azithromycin reduce amplitude suggesting they block I_{Na} or I_{Ca} (**Figure 14A**). In a clinical ECG, block of sodium current is demonstrated to slow conduction and widen the QRS complex (270). Clinical trials showed that COVID-19 patients given hydroxychloroquine or azithromycin had little change in their QRS complex, contrasting with our results(200). Furthermore, while several of the clinical trials did report prolongation of the QT interval, there were no reports of torsades de pointes. This is surprising, as we show several proarrhythmic

markers associated with torsades including EADs, ectopy and prolongation of APD90. These inconsistencies are likely due to differences in dosing. The peak plasma concentration with a 200mg dose hydroxychloroquine and a 400mg dose of azithromycin is 0.4 μ M and 0.5 μ M respectively(171, 230). We did not observe a reduction in the amplitude of the sodium spike until concentrations exceeding 1 μ M, suggesting QRS widening may not be observed until higher concentrations of drug are reached in the blood.

The amount of drug available for ion channel binding in the patient's blood compared to our assay should also be considered. In the blood, 30- 40% of hydroxychloroquine is protein bound, while azithromycin is less than 50% protein bound(67, 230). We exposed hiPSC-CM to the drugs using serum free media, therefore the amount of drug available for binding is likely much higher compared to patients.

Additionally, our system does not take into account the amount of drug that gets absorbed into other tissues as the field potential is only being measured from a monolayer of cardiomyocytes. Azithromycin is shown to be heavily sequestered in tissue, again suggesting that there are differences in drug concentration between the patient's blood and our assay(74, 270).

While clinical data is incredibly valuable, it can take several months to acquire as finding the appropriate cohort and sample size can take a long time. Our results took only 2 months to acquire and an additional month of analysis. Given that our results are consistent with clinical data examining the QT interval, we show that hiPSC-CM are a great model for quickly obtaining information regarding the potential cardiotoxic effects of a drug.

POSSIBLE MECHANISMS UNDERLYING FPD PROLONGATION IN hiPSC-CM

Human ether-a-go-go (hERG) is a potassium channel critical to cell repolarization (214, 257). Many drugs are known to block hERG including both chloroquine and loperamide(124, 128, 248). Block of this channel is demonstrated to prolong the APD90 in a cardiomyocyte or the QT interval in an ECG(39). In fact, hERG block is one of the major causes of drug induced QT prolongation(257). Therefore, hERG block seems to be the most likely cause of APD prolongation, however there could be other contributing factors.

We show that azithromycin and hydroxychloroquine both reduce the sodium depolarization spike (P_{max}) (**Figure 14A**). This observation could be explained through two mechanisms. Azithromycin is shown to be a sodium channel blocker, suggesting that the reduction in amplitude of the sodium spike could be due to sodium channel block(270). Another mechanism could be attributed to potassium channel block. The resting membrane potential in hiPSC-CM is suggested to be set by hERG as these cells lack I_{K1} (46). Therefore, hERG block could result in an increased resting membrane potential resulting in less sodium channels available for activation.

While we did observe the net effects of these drugs on FPD, identifying the mechanisms prolonging the FPD would be of great interest to our research. Chloroquine is demonstrated to block hERG and I_{K1} , however there is little peer-reviewed evidence examining the effects of azithromycin and hydroxychloroquine on these channels(13, 205, 212, 213, 248). The binding site of chloroquine on Kir2.x channels has been identified and involves both charged and aromatic residues(205). Furthermore, two aromatic residues (Y652 and F656) within the transmembrane domain of hERG are notorious for binding drugs and blocking the channel(167). We speculate that hydroxychloroquine may also bind to these residues causing block of hERG resulting in a prolonged FPD. In order to test this hypothesis, we could use patch clamp to assess the concentration of hydroxychloroquine that blocked hERG current in a heterologous

cell system. If current was blocked, the binding site could be determined using alanine scanning.

CLOSING

In this thesis, we advance our current understanding of K_{ATP} and present hiPSC-CM as a model to study the effects of repurposed drugs. We demonstrate a novel cholesterol dependent mechanism regulating SUR2 expression, suggesting that hypercholesterolemia might lead to poor cardiovascular health, in part by reducing K_{ATP} generation. We also show that hiPSC-CM express functional K_{ATP} channels, opening a new avenue to study the structure and function of the K_{ATP} channel. Finally, we demonstrated an assay using hiPSC-CM that can quickly assess the proarrhythmic capabilities of several drugs implicated in the COVID-19 pandemic and opioid epidemic.

REFERENCES

1. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement IV JP, Boyd AE, et al. 1995. Cloning of the β cell high-affinity sulfonylurea receptor: A regulator of insulin secretion. *Science* (80-.). 268(5209):423–26
2. Aittoniemi J, Fotinou C, Craig TJ, De Wet H, Proks P, Ashcroft FM. 2009. SUR1: A unique ATP-binding cassette protein that functions as an ion channel regulator
3. Akao M, Otani H, Horie M, Takano M, Kuniyasu A, et al. 1997. Myocardial ischemia induces differential regulation of K(ATP) channel gene expression in rat hearts. *J. Clin. Invest.* 100(12):3053–59
4. Baartscheer A, Schumacher CA, Wekker V, Verkerk AO, Veldkamp MW, et al. 2015. Dyscholesterolemia Protects Against Ischemia-Induced Ventricular Arrhythmias. *Circ. Arrhythmia Electrophysiol.* 8(6):1481–90
5. Babenko AP, Gonzalez G, Aguilar-Bryan L, Bryan J. 1998. Reconstituted Human Cardiac K ATP Channels . *Circ. Res.* 83(11):1132–43
6. Babenko AP, Gonzalez G, Bryan J. 2000. Pharmacology of Sulfonylurea Receptors SEPARATE DOMAINS OF THE REGULATORY SUBUNITS OF K ATP CHANNEL ISOFORMS ARE REQUIRED FOR SELECTIVE INTERACTION WITH K CHANNEL OPENERS*. *J. Biol. Chem.* 275(2):717–20
7. Baczkó I, Giles WR, Light PE. 2004. Pharmacological activation of plasma-membrane K ATP channels reduces reoxygenation-induced Ca^{2+} overload in cardiac myocytes via modulation of the diastolic membrane potential. *Br. J. Pharmacol.* 141(6):1059–67
8. Baczkó I, Jones L, McGuigan CF, Fox JEM, Gandhi M, et al. 2005. Plasma-membrane K. *FASEB J.* 26:1–26
9. Baczkó I, Jones L, McGuigan CF, Fox JEM, Gandhi M, et al. 2005. Plasma membrane KATP channel-mediated cardioprotection involves posthypoxic reductions in calcium overload and contractile dysfunction: Mechanistic insights into cardioplegia. *FASEB J.* 19(8):
10. Bansal P, Goyal A, Cusick A, Lahan S, Dhaliwal HS, et al. 2021. Hydroxychloroquine: a comprehensive review and its controversial role in coronavirus disease 2019. *Ann. Med.* 53(1):117–34
11. Baukowitz T, Schulte U, Oliver D, Herlitze S, Krauter T, et al. 1998. PIP₂ and PIP as determinants for ATP inhibition of K(ATP) channels. *Science* (80-.). 282(5391):1141–44

12. Baumann P, Poitry S, Roatti A, Baertschi AJ. 2002. Plasmalemmal KATP channels shape triggered calcium transients in metabolically impaired rat atrial myocytes. *Am. J. Physiol. - Hear. Circ. Physiol.* 283(6 52-6):2296–2305
13. Benavides-Haro DE, Sánchez-Chapula JA. 2000. Chloroquine blocks the background potassium current in guinea pig atrial myocytes. *Naunyn. Schmiedebergs. Arch. Pharmacol.* 361(3):311–18
14. Benjamin EJ, Muntner P, Alonso A, Bittencourt MS, Callaway CW, et al. 2019. Heart Disease and Stroke Statistics-2019 Update: A Report From the American Heart Association. *Circulation.* 139(10):e56–66
15. Billman GE. 2008. The cardiac sarcolemmal ATP-sensitive potassium channel as a novel target for anti-arrhythmic therapy. *Pharmacol. Ther.* 120(1):54–70
16. Blazeski A, Zhu R, Hunter DW, Weinberg SH, Boheler KR, et al. 2012. Electrophysiological and contractile function of cardiomyocytes derived from human embryonic stem cells. *Prog. Biophys. Mol. Biol.* 110(2–3):178–95
17. Brown MS, Goldstein JL. 1999. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci. U. S. A.* 96(20):11041–48
18. Brownstein CA, Towne MC, Luquette LJ, Harris DJ, Marinakis NS, et al. 2013. Mutation of KCNJ8 in a patient with Cantú syndrome with unique vascular abnormalities – Support for the role of K(ATP) channels in this condition. *Eur. J. Med. Genet.* 56(12):678–82
19. Buber J, Goldenberg I, Moss AJ, Wang PJ, McNitt S, et al. 2012. Reduction in life-threatening ventricular tachyarrhythmias in statin-treated patients with nonischemic cardiomyopathy enrolled in the MADIT-CRT (Multicenter Automatic Defibrillator Implantation Trial with Cardiac Resynchronization Therapy). *J. Am. Coll. Cardiol.* 60(8):749–55
20. Campbell JD, Proks P, Lippiat JD, Sansom MSP, Ashcroft FM. 2004. Identification of a functionally important negatively charged residue within the second catalytic site of the SUR1 nucleotide-binding domains
21. Cantú JM, Garcia-Cruz D, Sánchez-Corona J, Hernández A, Nazaré Z. 1982. A distinct osteochondrodysplasia with hypertrichosis-Individualization of a probable autosomal recessive entity. *Hum. Genet.* 60(1):36–41
22. Capel RA, Herring N, Kalla M, Yavari A, Mirams GR, et al. 2015. Hydroxychloroquine reduces heart rate by modulating the hyperpolarization-activated current If: Novel electrophysiological insights

and therapeutic potential. *Hear. Rhythm.* 12(10):2186–94

23. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M WT. 2005. MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics.* 21:2933–42
24. Cavalcanti AB, Zampieri FG, Rosa RG, Azevedo LCP, Veiga VC, et al. 2020. Hydroxychloroquine with or without Azithromycin in Mild-to-Moderate Covid-19. *N. Engl. J. Med.* 383(21):2041–52
25. Chan KW, Wheeler A, Csanády L. 2008. Sulfonylurea receptors type 1 and 2A randomly assemble to form heteromeric KATP channels of mixed subunit composition. *J. Gen. Physiol.* 131(1):43–58
26. Chittur S V, Sangster-Guity N, McCormick PJ. 2008. Histone deacetylase inhibitors: a new mode for inhibition of cholesterol metabolism. *BMC Genomics.* 9:
27. Chorin E, Wadhwani L, Magnani S, Dai M, Shulman E, et al. 2020. QT interval prolongation and torsade de pointes in patients with COVID-19 treated with hydroxychloroquine/azithromycin. *Hear. Rhythm.* 17(9):1425–33
28. Chutkow WA, Makielski JC, Nelson DJ, Burant CF, Fan Z. 1999. Alternative splicing of sur2 exon 17 regulates nucleotide sensitivity of the ATP-sensitive potassium channel. *J. Biol. Chem.* 274(19):13656–65
29. Chutkow WA, Pu J, Wheeler MT, Wada T, Makielski JC, et al. 2002. Episodic coronary artery vasospasm and hypertension develop in the absence of Sur2 KATP channels. *J. Clin. Invest.* 110(2):203–8
30. Chutkow WA, Samuel V, Hansen PA, Pu J, Valdivia CR, et al. 2001. Disruption of Sur2-containing K ATP channels enhances insulin-stimulated glucose uptake in skeletal muscle. *Proc. Natl. Acad. Sci. U. S. A.* 98(20):11760–64
31. Chutkow WA, Simon MC, Le Beau MM, Burant CF. 1996. Cloning, tissue expression, and chromosomal localization of SUR2, the putative drug-binding subunit of cardiac, skeletal muscle, and vascular K(ATP) channels. *Diabetes.* 45(1439–1445):
32. Clement IV JP, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, et al. 1997. Association and stoichiometry of K(ATP) channel subunits. *Neuron.* 18(5):827–38
33. Cole WC, McPherson CD, Sontag D. 1991. ATP-regulated K⁺ channels protect the myocardium against ischemia/reperfusion damage. *Circ. Res.*

34. Cook DL, Hales N. 1984. Intracellular ATP directly blocks K⁺ channels in pancreatic B-cells. *Nature*. 311(5983):271–73
35. Crawford RM, Jovanović S, Budas GR, Davies AM, Lad H, et al. 2003. Chronic mild hypoxia protects heart-derived H9c2 cells against acute hypoxia/reoxygenation by regulating expression of the SUR2A subunit of the ATP-sensitive K⁺ channel. *J. Biol. Chem.* 278(33):31444–55
36. Csonka C, Kupai K, Bencsik P, Görbe A, Pálóczi J, et al. 2014. Cholesterol-enriched diet inhibits cardioprotection by ATP-sensitive K⁺ channel activators cromakalim and diazoxide. *Am. J. Physiol. Circ. Physiol.* 306(3):H405–13
37. Cui Y, Gliblin JP, Clapp LH, Tinker A. 2001. A mechanism for ATP-sensitive potassium channel diversity: Functional coassembly of two pore-forming subunits. *Proc. Natl. Acad. Sci. U. S. A.* 98(2):729–34
38. Cui Y, Gliblin JP, Clapp LH, Tinker A. 2001. A mechanism for ATP-sensitive potassium channel diversity: Functional coassembly of two pore-forming subunits. *Proc. Natl. Acad. Sci.* 98(2):729–34
39. Curran ME, Splawski I, Timothy KW, Vincen GM, Green ED, Keating MT. 1995. A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome. *Cell*. 80(5):795–803
40. Dart BC, Standen NB. 1993. ADENOSINE-ACTIVATED POTASSIUM CURRENT IN SMOOTH MUSCLE CELLS ISOLATED FROM THE PIG CORONARY ARTERY. *J. Physiol.* 471:767–86
41. Davis MJ, Kim HJ, Zawieja SD, Castorena-Gonzalez JA, Gui P, et al. 2020. Kir6.1-dependent KATP channels in lymphatic smooth muscle and vessel dysfunction in mice with Kir6.1 gain-of-function. *J. Physiol.* 598(15):3107–27
42. Dhamoon AS, Jalife J. 2005. The inward rectifier current (I_{K1}) controls cardiac excitability and is involved in arrhythmogenesis. *Hear. Rhythm.* 2(3):316–24
43. Di Diego JM, Antzelevitch C. 1993. Pinacidil-induced electrical heterogeneity and extrasystolic activity in canine ventricular tissues does activation of atp-regulated potassium current promote phase 2 reentry? *Circulation*. 88(3):1177–89
44. Dickinson KEJ, Bryson CC, Cohen RB, Rogers L, Green DW, Atwal KS. 1997. Nucleotide regulation and characteristics of potassium channel opener binding to skeletal muscle membranes. *Mol. Pharmacol.*

45. Dörschner H, Brekardin E, Uhde I, Schwanstecher C, Schwanstecher M. 1999. Stoichiometry of sulfonylurea-induced ATP-sensitive potassium channel closure. *Mol. Pharmacol.* 55(6):1060–66
46. Doss MX, Di Diego JM, Goodrow RJ, Wu Y, Cordeiro JM, et al. 2012. Maximum diastolic potential of human induced pluripotent stem cell-derived cardiomyocytes depends critically on IKr. *PLoS One.* 7(7):
47. Du Q, Jovanović S, Clelland A, Sukhodub A, Budas G, et al. 2006. Overexpression of SUR2A generates a cardiac phenotype resistant to ischemia. *FASEB J.* 20(8):1131–41
48. Dunne MJ, Petersen OH. 1986. Intracellular ADP activates K⁺ channels that are inhibited by ATP in an insulin-secreting cell line. *FEBS Lett.* 208(1):59–62
49. Elcarpio JOB, Ahinski ANB, Zzo NIJI, Claycomb WC, Lanson NA, et al. 1998. HL-1 cells : A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc. Natl. Acad. Sci. U. S. A.* 95(6):2979–84
50. Elliott AC, Smith GL, Allen DG. 1989. Simultaneous measurements of action potential duration and intracellular ATP in isolated ferret hearts exposed to cyanide. *Circ. Res.* 64(3):583–91
51. Enkvetchakul D, Loussouarn G, Makhina E, Shyng SL, Nichols CG. 2000. The kinetic and physical basis of K(ATP) channel gating: Toward a unified molecular understanding. *Biophys. J.* 78(5):2334–48
52. Enkvetchakul D, Nichols CG. 2003. Gating Mechanism of KATP Channels: Function Fits Form. *J. Gen. Physiol.* 122(5):471–80
53. Epshtein Y, Chopra AP, Rosenhouse-Dantsker A, Kowalsky GB, Logothetis DE, Levitan I. 2009. Identification of a C-terminus domain critical for the sensitivity of Kir2.1 to cholesterol. *Proc. Natl. Acad. Sci. U. S. A.* 106(19):8055–60
54. Fan Z, Makielski JC. 1997. Anionic phospholipids activate ATP-sensitive potassium channels. *J. Biol. Chem.* 272(9):5388–95
55. Fatima A, Xu G, Shao K, Papadopoulos S, Lehmann M, et al. 2011. In vitro modeling of ryanodine receptor 2 dysfunction using human induced pluripotent stem cells. *Cell. Physiol. Biochem.* 28(4):579–92
56. Fatima N, Cohen DC, Sukumar G, Sissung TM, Schooley Jr. JF, et al. 2015. Histone deacetylase inhibitors modulate KATP subunit transcription in HL-1 cardiomyocytes through effects on cholesterol homeostasis. *Front Pharmacol.* 6(August):168

57. Fatima N, Schooley JF, Claycomb WC, Flagg TP. 2012. Promoter DNA methylation regulates murine SUR1 (Abcc8) and SUR2 (Abcc9) expression in HL-1 cardiomyocytes. *PLoS One*. 7(7):1–10
58. Feaster TK, Cadar AG, Wang L, Williams CH, Chun YW, et al. 2015. A method for the generation of single contracting human-induced pluripotent stem cell-derived cardiomyocytes. *Circ. Res.* 117(12):995–1000
59. Fedorov V V., Glukhov A V., Ambrosi CM, Kosteki G, Chang R, et al. 2011. Effects of KATP channel openers diazoxide and pinacidil in coronary-perfused atria and ventricles from failing and non-failing human hearts. *J. Mol. Cell. Cardiol.* 51(2):215–25
60. Ferdinandy P, Szilvassy Z, Horváth LI, Csont T, Csonka C, et al. 1997. Loss of pacing-induced preconditioning in rat hearts: Role of nitric oxide and cholesterol-enriched diet. *J. Mol. Cell. Cardiol.* 29(12):3321–33
61. Ficker E, Taglialatela M, Wible BA, Henley CM, Brown AM. 1994. Spermine and spermidine as gating molecules for inward rectifier K⁺ channels. *Science* (80-). 266(5187):1068–72
62. Finzer P, Kuntzen C, Soto U, Zur Hausen H, Rösl F. 2001. Inhibitors of histone deacetylase arrest cell cycle and induce apoptosis in cervical carcinoma cells circumventing human papillomavirus oncogene expression. *Oncogene*. 20(35):4768–76
63. Flagg TP, Enkvetchakul D, Koster JC, Nichols CG. 2010. Muscle K ATP Channels: Recent Insights to Energy Sensing and Myoprotection. *Physiol. Rev.* 90(3):799–829
64. Flagg TP, Kurata HT, Masia R, Caputa G, Magnuson MA, et al. 2008. Differential structure of atrial and ventricular KATP: Atrial KATP channels require SUR1. *Circ. Res.* 103(12):1458–65
65. Flanagan SE, Clauin S, Bellanné-Chantelot C, De Lonlay P, Harries LW, et al. 2009. Update of mutations in the genes encoding the pancreatic beta-cell KATP channel subunits Kir6.2 (KCNJ11) and sulfonylurea receptor 1 (ABCC8) in diabetes mellitus and hyperinsulinism. *Hum. Mutat.* 30(2):170–80
66. Foster MN, Coetzee WA. 2016. K ATP Channels in the Cardiovascular System. *Physiol. Rev.* 96:177–252
67. Furst DE. 1996. Pharmacokinetics of hydroxychloroquine and chloroquine during treatment of rheumatic diseases. *Lupus*. 5 Suppl 1:S11-5
68. Gao J, Tian Z, Yang X. 2020. Breakthrough: Chloroquine phosphate has shown apparent efficacy in treatment of COVID-19 associated pneumonia

in clinical studies. *Biosci. Trends.* 14(1):72–73

69. Garg P, Garg V, Shrestha R, Sanguinetti MC, Kamp TJ, Wu JC. 2018. Human induced pluripotent stem cell-derived cardiomyocytes as models for cardiac channelopathies: A primer for non-electrophysiologists. *Circ. Res.* 123(2):224–43
70. Gaspar JA, Doss MX, Hengstler JG, Cadenas C, Hescheler J, Sachinidis A. 2014. Unique metabolic features of stem cells, cardiomyocytes, and their progenitors. *Circ. Res.* 114(8):1346–60
71. Gautret P, Lagier J, Parola P, Hoang VT. 2020. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-label non-randomized clinical trial ☆. *Int. J. Antimicrob. Agents.* 56(1):
72. Geiger RM, Klein MG, Fatima N, Goldstein RE, Krantz MJ, et al. 2020. Rapid Assessment of Proarrhythmic Potential Using Human iPSC-Derived Cardiomyocytes. *JACC Clin. Electrophysiol.* 6(14):1860–62
73. Geleris J, Sun Y, Platt J, Zucker J, Baldwin M, et al. 2020. Observational Study of Hydroxychloroquine in Hospitalized Patients with Covid-19. *N. Engl. J. Med.* 382(25):2411–18
74. Girard AE, Girard D, English AR, Gootz TD, Cimochoowski CR, et al. 1987. Pharmacokinetic and in vivo studies with azithromycin (CP-62,993), a new macrolide with an extended half-life and excellent tissue distribution. *Antimicrob. Agents Chemother.* 31(12):1948–54
75. Golino P, Maroko PR, Carew TE. 1987. Efficacy of platelet depletion in counteracting the detrimental effect of acute hypercholesterolemia on infarct size and the no-reflow phenomenon in rabbits undergoing coronary artery occlusion-reperfusion. *Circulation.* 76(1):173–80
76. Görbe A, Varga Z V, Kupai K, Bencsik P, Kocsis GF, et al. 2011. Cholesterol diet leads to attenuation of ischemic preconditioning-induced cardiac protection: The role of connexin 43. *Am. J. Physiol. - Hear. Circ. Physiol.* 300(5):H1907–13
77. Goversen B, van der Heyden MAG, van Veen TAB, de Boer TP. 2018. The immature electrophysiological phenotype of iPSC-CMs still hampers in vitro drug screening: Special focus on IK1. *Pharmacol. Ther.* 183(October 2017):127–36
78. Gribble FM, Ashcroft FM. 1999. Differential sensitivity of beta-cell and extrapancreatic K(ATP) channels to gliclazide. *Diabetologia.* 42(7):845–48
79. Gribble FM, Tucker SJ, Haug T, Ashcroft FM. 1998. MgATP activates the β cell KATP channel by interaction with its SUR1 subunit. *Proc. Natl. Acad.*

Sci. U. S. A. 95(12):7185–90

80. Gross GJ, Auchampach JA. 1992. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ. Res.* 70(2):223–33
81. Gross GJ, Peart JN. 2003. KATP channels and myocardial preconditioning: An update. *Am. J. Physiol. - Hear. Circ. Physiol.* 285(3 54-3):
82. Gumina RJ, O’Cochlain DF, Kurtz CE, Bast P, Pucar D, et al. 2007. KATP channel knockout worsens myocardial calcium stress load in vivo and impairs recovery in stunned heart. *Am. J. Physiol. - Hear. Circ. Physiol.* 292(4):1706–13
83. Halperin RO, Sesso HD, Ma J, Buring JE, Stampfer MJ, Gaziano JM. 2006. Dyslipidemia and the risk of incident hypertension in men. *Hypertension.* 47(1):45–50
84. Hambrock A, Löffler-Walz C, Kurachi Y, Quast U. 1998. Mg²⁺ and ATP dependence of K(ATP) channel modulator binding to the recombinant sulphonylurea receptor, SUR2B. *Br. J. Pharmacol.* 125(3):577–83
85. Handy DE, Castro R, Loscalzo J. 2011. Epigenetic modifications: Basic mechanisms and role in cardiovascular disease. *Circulation.* 123(19):2145–56
86. Harada T, Hideshima T, Anderson KC. 2016. Histone deacetylase inhibitors in multiple myeloma: from bench to bedside. *Int. J. Hematol.* 104(3):300–309
87. Harakalova M, Van Harssel JJT, Terhal PA, Van Lieshout S, Duran K, et al. 2012. Dominant missense mutations in ABCC9 cause Cantú syndrome. *Nat. Genet.* 44(7):793–96
88. Hawkins JL, Robbins MD, Warren LC, Xia D, Petras SF, et al. 2008. Pharmacologic inhibition of site 1 protease activity inhibits sterol regulatory element-binding protein processing and reduces lipogenic enzyme gene expression and lipid synthesis in cultured cells and experimental animals. *J. Pharmacol. Exp. Ther.* 326(3):801–8
89. Hayes HB, Nicolini AM, Arrowood CA, Chvatal SA, Wolfson DW, et al. 2019. Novel method for action potential measurements from intact cardiac monolayers with multiwell microelectrode array technology. *Sci. Rep.* 9(1):1–13
90. Heginbotham L, Lu Z, Abramson T, MacKinnon R. 1994. Mutations in the K⁺ channel signature sequence. *Biophys. J.* 66(4):1061–67

91. Hernández-Sánchez C, Ito Y, Ferrar J, Reitman M, LeRoith D. 1999. Characterization of the mouse sulfonylurea receptor 1 promoter and its regulation. *J. Biol. Chem.* 274(26):18261–70
92. Hernández D, Millard R, Sivakumaran P, Wong RCB, Crombie DE, et al. 2016. Electrical stimulation promotes cardiac differentiation of human induced pluripotent stem cells. *Stem Cells Int.* 2016:
93. Higgins CF. 1992. ABC Transporters: From microorganisms to man
94. Hoekstra M, Mummery CL, Wilde AAM, Bezzina CR, Verkerk AO. 2012. Induced pluripotent stem cell derived cardiomyocytes as models for cardiac arrhythmias. *Front. Physiol.* 3 AUG(August):1–14
95. Hopkins WF, Fatherazi S, Peter-Riesch B, Corkey BE, Cook DL. 1992. Two sites for adenine-nucleotide regulation of ATP-sensitive potassium channels in mouse pancreatic β -cells and HIT cells. *J. Membr. Biol.*
96. Horie M, Irisawa H, Noma A. 1987. Voltage-dependent magnesium block of adenosine-triphosphate-sensitive potassium channel in guinea-pig ventricular cells. *J. Physiol.* 387(1):251–72
97. Horton JD, Goldstein JL, Brown MS. 2002. SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver
98. Hough E, Beech DJ, Sivaprasadarao A. 2000. Identification of molecular regions responsible for the membrane trafficking of Kir6.2. *Pflugers Arch. Eur. J. Physiol.* 440(3):481–87
99. Hu H, Sato T, Seharaseyon J, Liu Y, Johns DC, et al. 1999. Pharmacological and histochemical distinctions between molecularly defined sarcolemmal K(ATP) channels and native cardiac mitochondrial K(ATP) channels. *Mol. Pharmacol.* 55(6):1000–1005
100. Huang Y, McClenaghan C, Harter TM, Hinman K, Halabi CM, et al. 2018. Cardiovascular consequences of KATP overactivity in Cantu syndrome. *JCI insight.* 3(15):1–16
101. Huang Y, Walker KE, Hanley F, Narula J, Houser SR, Tulenko TN. 2004. Cardiac Systolic and Diastolic Dysfunction after a Cholesterol-Rich Diet. *Circulation.* 109(1):97–102
102. Huopio H, Otonkoski T, Vauhkonen I, Reimann F, Ashcroft FM, Laakso M. 2003. A new subtype of autosomal dominant diabetes attributable to a mutation in the gene for sulfonylurea receptor 1. *Lancet.* 361(9354):301–7
103. Huysmans FTM, Thien T, Koene RA. 1983. Acute Treatment of Hypertension With Slow Infusion of Diazoxide. *Arch. Intern. Med.*

104. Hyun JJ, Lee HS, Kim KS, Kim YK, Yoon D, Sahng WP. 2008. Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2. *J. Lipid Res.* 49(2):399–409
105. Inagaki N, Gonoi T, Clement IV JP, Namba N, Inazawa J, et al. 1995. Reconstitution of IKATP: An Inward Rectifier Subunit Plus the Sulfonylurea Receptor. *Science* (80-.). 270:1166–69
106. Inagaki N, Gonoi T, Clement IV JP, Namba N, Inazawa J, et al. 1995. Reconstitution Of Ikatp: An Inward Rectifier Subunit Plus the Sulfonylurea Receptor. *Science* (80-.). 270(November 1995):1166–69
107. Inagaki N, Gonoi T, Clement IV JP, Wang CZ, Aguilar-Bryan L, et al. 1996. A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K⁺ channels. *Neuron*. 16:1011–17
108. Inagaki N, Inazawa J, Seino S. 1995. cDNA sequence, gene structure, and chromosomal localization of the human ATP-sensitive potassium channel, ukatp-1, gene (KCNJ8). *Genomics*. 30(1):102–4
109. Isidoro Tavares N, Philip-Couderc P, Papageorgiou I, Baertschi AJ, Lerch R, Montessuit C. 2007. Expression and function of ATP-dependent potassium channels in late post-infarction remodeling. *J. Mol. Cell. Cardiol.* 42(6):1016–25
110. Isomoto S, Kondo C, Yamada M, Matsumoto S, Higashiguchi O, et al. 1996. A novel sulfonylurea receptor forms with BIR (Kir6.2) a smooth muscle type ATP-sensitive K⁺ channel. *J. Biol. Chem.*
111. Itzhaki I, Maizels L, Huber I, Gepstein A, Arbel G, et al. 2012. Modeling of catecholaminergic polymorphic ventricular tachycardia with patient-specific human-induced pluripotent stem cells. *J. Am. Coll. Cardiol.* 60(11):990–1000
112. Jacobson MS, Ambudkar IS, Young EP, Naseem SM, Heald FP, Shamoo AE. 1985. Cardiac sarcoplasmic reticulum Effects of an atherogenic diet during the neonatal and juvenile period. *Atherosclerosis*. 55(1):81–91
113. Jennings RB. 2013. Historical perspective on the pathology of myocardial ischemia/reperfusion injury. *Circ. Res.* 113(4):428–38
114. JENNINGS RB, SOMMERS HM, SMYTH GA, FLACK HA, LINN H. 1960. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Arch. Pathol.* 70:68–78
115. Jeon T Il, Osborne TF. 2012. SREBPs: Metabolic integrators in physiology and metabolism

116. Jeong HJ, Lee H-S, Kim K-S, Kim Y-K, Yoon D, Park SW. 2008. Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2. *J. Lipid Res.* 49(2):399–409
117. Johannesen L, Vicente J, Mason JW, Sanabria C, Waite-Labott K, et al. 2014. Differentiating drug-induced multichannel block on the electrocardiogram: Randomized study of dofetilide, quinidine, ranolazine, and verapamil. *Clin. Pharmacol. Ther.* 96(5):549–58
118. Joseph L, Robinson JG. 2015. Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Inhibition and the Future of Lipid Lowering Therapy. *Prog. Cardiovasc. Dis.* 58(1):19–31
119. Jovanovic A, Jovanović S, Lorenz E, Terzic A. 1998. Recombinant Cardiac ATP-Sensitive K_h Channel Subunits Confer Resistance To Chemical Hypoxia-Reoxygenation Injury. *Circulation*, pp. 1548–55
120. Jovanović N, Jovanović S, Jovanović A, Terzic A. 1999. Gene delivery of Kir6.2/SUR2A in conjunction with pinacidil handles intracellular Ca²⁺ homeostasis under metabolic stress. *FASEB J.* 13(8):923–29
121. Jung CB, Moretti A, Mederos y Schnitzler M, Iop L, Storch U, et al. 2012. Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of catecholaminergic polymorphic ventricular tachycardia. *EMBO Mol. Med.* 4(3):180–91
122. Kakei M, Kelly RP, Ashcroft SJH, Ashcroft FM. 1986. The ATP-sensitivity of K⁺ channels in rat pancreatic B-cells is modulated by ADP. *FEBS Lett.*
123. Kamp TJ, Hamdan MH, January CT. 2020. Chloroquine or Hydroxychloroquine for COVID-19: Is Cardiotoxicity a Concern? *J. Am. Heart Assoc.* 9(12):e016887
124. Kang J, Compton DR, Vaz RJ, Rampe D. 2016. Proarrhythmic mechanisms of the common anti-diarrheal medication loperamide: revelations from the opioid abuse epidemic. *Naunyn. Schmiedeberg's Arch. Pharmacol.* 389(10):1133–37
125. Karakikes I, Ameen M, Termglinchan V, Wu JC. 2015. Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes: Insights into Molecular, Cellular, and Functional Phenotypes. *Circ. Res.* 117(1):80–88
126. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, et al. 2001. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J. Clin. Invest.* 108(3):407–14
127. Kisilevsky E, Kohly RP, Margolin EA. 2019. Dilated and tortuous retinal

- vessels as a sign of Cantu syndrome. *Ophthalmic Genet.* 40(5):453–54
128. Klein MG, Haigney MCP, Mehler PS, Fatima N, Flagg TP, Krantz MJ. 2016. Potent Inhibition of hERG Channels by the Over-the-Counter Antidiarrheal Agent Loperamide. *JACC Clin. Electrophysiol.* 2(7):784–89
 129. Knopp A, Thierfelder S, Koopmann R, Biskup C, Böhle T, Benndorf K. 1999. Anoxia generates rapid and massive opening of K(ATP) channels in ventricular cardiac myocytes. *Cardiovasc. Res.* 41(3):629–40
 130. Kocić I, Konstański Z, Kaminski M, Dworakowska D, Dworakowski R. 1999. Experimental hyperlipidemia prevents the protective effect of ischemic preconditioning on the contractility and responsiveness to phenylephrine of rat-isolated stunned papillary muscle. *Gen. Pharmacol.* 33(3):213–19
 131. Koster JC, Marshall BA, Ensor N, Corbett JA, Nichols CG. 2000. Targeted overactivity of β cell K(ATP) channels induces profound neonatal diabetes. *Cell.* 100(6):645–54
 132. Krantz MJ, Palmer RB, Haigney MCP. 2021. Cardiovascular Complications of Opioid Use: JACC State-of-the-Art Review. *J. Am. Coll. Cardiol.* 77(2):205–23
 133. Krauter T, Ruppersberg JP, Baukrowitz T. 2001. Phospholipids as modulators of KATP channels: Distinct mechanisms for control of sensitivity to sulphonylureas, K⁺ channel openers, and ATP. *Mol. Pharmacol.* 59(5):1086–93
 134. Krul IM, Sc M, Lugtenburg PJ, Ph D, Leeuwen FE Van, Ph D. 2010. Patient-Specific Induced Pluripotent Stem-Cell Models for Long-QT Syndrome. *N. Engl. J. Med.* 363(15):687–96
 135. Kubota I, Yamaki M, Shibata T, Ikeno E, Hosoya Y, Tomoike H. 1993. Role of ATP-Sensitive K⁺ Channel on ECG ST Segment Elevation During a Bout of Myocardial Ischemia A Study on Epicardial Mapping in Dogs. *Circulation.* 88(4):1845–51
 136. Kyriakides ZS, Psychari S, Iliodromitis EK, Kolettis TM, Sbarouni E, Kremastinos DT. 2002. Hyperlipidemia prevents the expected reduction of myocardial ischemia on repeated balloon inflations during angioplasty. *Chest.* 121(4):1211–15
 137. Lederer WJ, Nichols CG. 1989. Nucleotide modulation of the activity of rat heart ATP-sensitive K⁺ channels in isolated membrane patches. *J. Physiol.* 419(1):193–211
 138. Lederer WJ, Nichols CG, Smith GL. 1989. The mechanism of early

- contractile failure of isolated rat ventricular myocytes subjected to complete metabolic inhibition. *J. Physiol.* 413(1):329–49
139. León DD De, Stanley CA. 2020. Permanent Neonatal Diabetes Mellitus Summary Genetic counseling Diagnosis Suggestive Findings. *Gene Rev.*, pp. 1–30
 140. Leventhal AR, Chen W, Tall AR, Tabas I. 2001. Acid Sphingomyelinase-deficient Macrophages Have Defective Cholesterol Trafficking and Efflux. *J. Biol. Chem.* 276(48):44976–83
 141. Levin MD, Singh GK, Zhang HX, Uchida K, Kozel BA, et al. 2016. KATP channel gain-of-function leads to increased myocardial L-type Ca^{2+} current and contractility in Cantu syndrome. *Proc. Natl. Acad. Sci. U. S. A.* 113(24):6773–78
 142. Levitan I, Singh DK, Rosenhouse-Dantsker A. 2014. Cholesterol binding to ion channels. *Front. Physiol.* 5(65):1–14
 143. Li A, Knutsen RH, Zhang H, Osei-Owusu P, Moreno-Dominguez A, et al. 2013. Hypotension due to Kir6.1 gain-of-function in vascular smooth muscle. *J. Am. Heart Assoc.* 2(4):1–13
 144. Li R a, Leppo M, Miki T, Seino S, Marbán E. 2000. Molecular Basis of Electrocardiographic ST-Segment Elevation Ronald A. Li, Michelle Leppo, Takashi Miki, Susumu Seino and Eduardo Marbán Circulation Research 2000, 87:837-839. *Animals*, pp. 837–39
 145. Li RA, Leppo M, Miki T, Seino S, Marbán E. 2000. Molecular Basis of Electrocardiographic ST-Segment Elevation. *Circ. Res.* 87:837–39
 146. Li RA, Leppo M, Miki T, Seino S, Marbán E. 2000. Molecular basis of electrocardiographic ST-segment elevation. *Circ. Res.* 87(10):837–39
 147. Lin Y, Linask KL, Mallon B, Johnson K, Klein M, et al. 2016. Heparin Promotes Cardiac Differentiation of Human Pluripotent Stem Cells in Chemically Defined Albumin-Free Medium, Enabling Consistent Manufacture of Cardiomyocytes. *Stem Cells Trans Med.* 3:527–38
 148. Lopatin AN, Makhlna EN, Nichols CG. 1994. Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature.* 372:366–69
 149. Lorenz E, Terzic A. 1999. Physical Association Between Recombinant Cardiac ATP-sensitive K^{+} Channel Subunits Kir6.2 and SUR2A. *J. Mol. Cell. Cardiol.* 31(2):425–34
 150. Loussouarn G, Marton LJ, Nichols CG. 2005. Molecular basis of inward

rectification: Structural features of the blocker defined by extended polyamine analogs. *Mol. Pharmacol.* 2(68):298–304

151. Ma J, Guo L, Fiene SJ, Anson BD, Thomson JA, et al. 2011. High purity human-induced pluripotent stem cell-derived cardiomyocytes: Electrophysiological properties of action potentials and ionic currents. *Am. J. Physiol. - Hear. Circ. Physiol.* 301(5):2006–17
152. MacGregor GG, Dong K, Vanoye CG, Tang L, Giebisch G, Hebert SC. 2002. Nucleotides and phospholipids compete for binding to the C terminus of KATP channels. *Proc. Natl. Acad. Sci. U. S. A.* 99(5):2726–31
153. Maczewski M, Maczewska J. 2006. Hypercholesterolemia Exacerbates Ventricular Remodeling in the Rat Model of Myocardial Infarction. *J. Card. Fail.* 12(5):399–405
154. Magañ MM, Osborne TF. 1996. Two Tandem Binding Sites for Sterol Regulatory Element Binding Proteins Are Required for Sterol Regulation of Fatty-acid Synthase Promoter* Downloaded from. *J. Biol. Chem.* 271(51):32689–94
155. Masia R, Nichols CG. 2008. Functional clustering of mutations in the dimer interface of the nucleotide binding folds of the sulfonylurea receptor. *J. Biol. Chem.* 283(44):30322–29
156. Matsuda H, Saigusa A, Irisawa H. 1987. Ohmic conductance through the inwardly rectifying K channel and blocking by internal Mg²⁺. *Nature.* 325:156–59
157. Matsuoka T, Matsushita K, Katayama Y, Fujita A, Inageda K, et al. 2000. C-terminal tails of sulfonylurea receptors control ADP-induced activation and diazoxide modulation of ATP-sensitive K⁺ channels. *Circ. Res.* 87(10):873–80
158. Mazzanti A, Briani M, Kukavica D, Bulian F, Marelli S, et al. 2020. Association of Hydroxychloroquine with QTc Interval in Patients with COVID-19. *Circulation*, pp. 513–15
159. McKinsey TA. 2012. Therapeutic Potential for HDAC Inhibitors in the Heart. *Annu. Rev. Pharmacol. Toxicol.* 52:303–19
160. McKinsey TA. 2012. Therapeutic Potential for HDAC Inhibitors in the Heart. *Annu. Rev. Pharmacol. Toxicol.* 52(1):303–19
161. McKinsey TA. 2012. Therapeutic Potential for HDAC Inhibitors in the Heart. *Annu. Rev. Pharmacol. Toxicol.* 52(1):303–19
162. MCPHERSON C, PIERCE G, COLE W. 1993. Ischemic cardioprotection

by ATP-sensitive K. *Am. J. Physiol. Heart Circ. Physiol.* 34(5):

163. McTaggart JS, Clark RH, Ashcroft FM. 2010. The role of the KATP channel in glucose homeostasis in health and disease: More than meets the islet. *J. Physiol.* 588(17):3201–9
164. Messenger AG, Rundegren J. 2004. Minoxidil: Mechanisms of action on hair growth
165. Mikhailov M V., Mikhailova EA, Ashcroft SJH. 2001. Molecular structure of the glibenclamide binding site of the β -cell KATP channel. *FEBS Lett.* 499(1–2):154–60
166. Miki T, Suzuki M, Shibasaki T, Uemura H, Sato T, et al. 2002. Mouse model of Prinzmetal angina by disruption of the inward rectifier Kir6.1. *Nat. Med.* 8:466–72
167. Mitcheson JS, Chen J, Lin M, Culberson C, Sanguinetti MC. 2000. A structural basis for drug-induced long QT syndrome. *Proc. Natl. Acad. Sci. U. S. A.* 97(22):12329–33
168. Miyoshi Y, Nakaya Y. 1991. ANGIOTENSIN II BLOCKS ATP-SENSITIVE PORCINE CORONARY ARTERY SMOOTH K⁺ CHANNELS IN MUSCLE CELLS. *Biochem. Biophys. Res. Commun.* 181(2):700–706
169. Miyoshi Y, Wakatsuki T, Nomura M, Saito K, Nakaya Y, Inoue I. 1992. Endothelin blocks ATP-sensitive K⁺ channels and depolarizes smooth muscle cells of porcine coronary artery. *Jpn. J. Pharmacol.* 58(SUPPL. 2):612–16
170. Moreau C, Jacquet H, Prost A-L, Dhahan N, Vivaudou M. 2000. The molecular basis of the specificity of action of KATP channel openers. *EMBO J.* 19(24):6644–51
171. Morrisette T, Lodise TP, Scheetz MH, Goswami S, Pogue JM, Rybak MJ. 2020. The Pharmacokinetic and Pharmacodynamic Properties of Hydroxychloroquine and Dose Selection for COVID-19: Putting the Cart Before the Horse. *Infect. Dis. Ther.* 9(3):561–72
172. Morrissey A, Rosner E, Lanning J, Parachuru L, Dhar Chowdhury P, et al. 2005. Immunolocalization of K ATP channel subunits in mouse and rat cardiac myocytes and the coronary vasculature. *BMC Physiol.* 5:1–18
173. Mummery CL, Zhang J, Ng ES, Elliott DA, Elefanty AG, Kamp TJ. 2012. Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: A methods overview. *Circ. Res.* 111(3):344–58

174. Murase K, Morrison KL, Tam PY, Stafford RL, Journak F, Weiss GA. 2003. EF-Tu Binding Peptides Identified, Dissected, and Affinity Optimized by Phage Display GDP conformation of EF-Tu. Because EF-Tu is abundant in the cell, other functions have been suggested, particularly during periods of cellular stress when protein synthesis is down. *Chem. Biol.* 10:161–68
175. Murry CE, Jennings RB, Reimer KA. 1986. Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation.* 74(5):1124–36
176. Nelson MT, Patlak JB, Worley JF, Standen NB. 1990. Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am. J. Physiol. - Cell Physiol.* 259(1 28-1):
177. Nelson MT, Quayle JM. 1995. Physiological roles and properties of potassium channels in arterial smooth muscle. *Am. J. Physiol.* 268(4):C799–822
178. Nelson MT, Standen N, Brayden JE, Worley JF. 1988. Noradrenaline contracts arteries by activating voltage-dependent calcium channels. *Nature.* 336(01):382–85
179. Nichols CG, Lederer WJ. 1990. The regulation of ATP-sensitive K⁺ channel activity in intact and permeabilized rat ventricular myocytes. *J. Physiol.* 423(1):91–110
180. Nichols CG, Lopatin AN. 1997. Inward rectifier potassium channels. *Annu. Rev. Physiol.* 59:171–91
181. Nichols CG, Ripoll C, Lederer WJ. 1991. ATP-Sensitive potassium channel modulation of the guinea pig ventricular action potential and contraction. *Circ. Res.* 68:280–87
182. Nichols CG, Shyng SL, Nestorowicz A, Glaser B, Clement IV JP, et al. 1996. Adenosine diphosphate as an intracellular regulator of insulin secretion. *Science (80-.).* 272(5269):1785–87
183. Nichols CG, Singh GK, Grange DK. 2013. KATP channels and cardiovascular disease: Suddenly a syndrome. *Circ. Res.* 112(7):1059–72
184. Noma A. 1983. ATP-regulated K⁺ Channels in cardiac muscle. *Nature.* 305:147–48
185. Noonan AM, Eisch RA, Liewehr DJ, Sissung TM, Venzon DJ, et al. 2013. Electrocardiographic studies of romidepsin demonstrate its safety and identify a potential role for KATP channel. *Clin. Cancer Res.* 19(11):3095–3104

186. Nunes MJ, Moutinho M, Gama MJ, Rodrigues CMP, Rodrigues E. 2013. Histone Deacetylase Inhibition Decreases Cholesterol Levels in Neuronal Cells by Modulating Key Genes in Cholesterol Synthesis, Uptake and Efflux. *PLoS One*. 8(1):
187. Oesterle A, Laufs U, Liao JK. 2017. Pleiotropic Effects of Statins on the Cardiovascular System
188. Osipov RM, Bianchi C, Feng J, Clements RT, Liu Y, et al. 2009. Effect of hypercholesterolemia on myocardial necrosis and apoptosis in the setting of ischemia-reperfusion. *Circulation*. 120:S22–30
189. Parish S, Offer A, Clarke R, Hopewell JC, Hill MR, et al. 2012. Lipids and lipoproteins and risk of different vascular events in the MRC/BHF heart protection study. *Circulation*. 125(20):2469–78
190. Park HJ, Georgescu SP, Du C, Madias C, Aronovitz MJ, et al. 2008. Parasympathetic response in chick myocytes and mouse heart is controlled by SREBP. *J. Clin. Invest*. 118(1):259–71
191. Pearson ER, Flechtner I, Njølstad PR, Malecki MT, Flanagan SE, et al. 2006. Switching from Insulin to Oral Sulfonylureas in Patients with Diabetes Due to Kir6.2 Mutations. *N. Engl. J. Med*. 355(5):467–77
192. Philip-Couderc P, Tavares NI, Roatti A, Lerch R, Montessuit C, Baertschi AJ. 2008. Forkhead transcription factors coordinate expression of myocardial KATP channel subunits and energy metabolism. *Circ. Res*. 102(2):20–35
193. Philip-Couderc P, Tavares NI, Roatti A, Lerch R, Montessuit C, Baertschi AJ. 2008. Forkhead transcription factors coordinate expression of myocardial KATP channel subunits and energy metabolism. *Circ. Res*. 102(2):20–35
194. Pipalia NH, Cosner CC, Huang A, Chatterjee A, Bourbon P, et al. 2011. Histone deacetylase inhibitor treatment dramatically reduces cholesterol accumulation in Niemann-Pick type C1 mutant human fibroblasts. *Proc. Natl. Acad. Sci*. 108(14):5620–25
195. Pitts KR, Toombs CF. 2004. Coverslip hypoxia: A novel method for studying cardiac myocyte hypoxia and ischemia in vitro. *Am. J. Physiol. - Hear. Circ. Physiol*. 287(4 56-4):1801–12
196. Quasthoff S, Spuler A, Lehmann-Horn F, Grafe P. 1989. Cromakalim, pinacidil and RP 49356 activate a tolbutamide-sensitive K⁺ conductance in human skeletal muscle fibres. *Pflugers Arch. Eur. J. Physiol*. 414:S179–80
197. Quayle JM, Bonev AD, Brayden JE, Nelson MT. 1995. Pharmacology of

ATP-sensitive K⁺ currents in smooth muscle cells from rabbit mesenteric artery. *Am. J. Physiol. - Cell Physiol.*

198. Quianzon CCL, Cheikh IE. 2012. History of current non-insulin medications for diabetes mellitus. *J. Community Hosp. Intern. Med. Perspect.* 2(3):19081
199. Quinn K V., Cui Y, Giblin JP, Clapp LH, Tinker A. 2003. Do anionic phospholipids serve as cofactors or second messengers for the regulation of activity of cloned ATP-sensitive K⁺ channels? *Circ. Res.* 93(7):646–55
200. Ramireddy A, Chugh H, Reinier K, Ebinger J, Park E, et al. 2020. Experience With Hydroxychloroquine and Azithromycin in the Coronavirus Disease 2019 Pandemic: Implications for QT Interval Monitoring. *J. Am. Heart Assoc.* 9(12):e017144
201. Ranki HJ, Budas GR, Crawford RM, Jovanovi A. 2001. Gender-specific difference in cardiac ATP-sensitive K⁺ channels. *J. Am. Coll. Cardiol.* 38(3):906–15
202. Reimer KA, Murry CE, Yamasawa I, Hill ML, Jennings RB. 1986. Four brief periods of myocardial ischemia cause no cumulative ATP loss or necrosis. *Am. J. Physiol. Circ. Physiol.* 251(6):H1306–15
203. Ribalet B, John SA, Xie LH, Weiss JN. 2005. Regulation of the ATP-sensitive K channel Kir6.2 by ATP and PIP₂. *J. Mol. Cell. Cardiol.* 39(1):71–77
204. Robertson DW, Steinberg MI. 1990. Potassium channel modulators: scientific applications and therapeutic promise. *J. Med. Chem.* 33(6):1529–41
205. Rodríguez-Menchaca AA, Navarro-Polanco RA, Ferrer-Villada T, Rupp J, Sachse FB, et al. 2008. The molecular basis of chloroquine block of the inward rectifier Kir2.1 channel. *Proc. Natl. Acad. Sci. U. S. A.* 105(4):1364–68
206. Romanenko VG, Fang Y, Byfield F, Travis AJ, Vandenberg CA, et al. 2004. Cholesterol sensitivity and lipid raft targeting of Kir2.1 channels. *Biophys. J.* 87(6):3850–61
207. Russ U, Hambrock A, Artunc F, Löffler-Walz C, Horio Y, et al. 1999. Coexpression with the inward rectifier K⁺ channel Kir6.1 increases the affinity of the vascular sulfonylurea receptor SUR2B for glibenclamide. *Mol. Pharmacol.* 56(5):955–61
208. Russ U, Lange U, Löffler-Walz C, Hambrock A, Quast U. 2001. Interaction of the sulfonylthiourea HMR 1833 with sulfonylurea receptors and

- recombinant ATP-sensitive K⁺ channels: Comparison with glibenclamide. *J. Pharmacol. Exp. Ther.* 299(3):1049–55
209. Sabatine MS. 2019. PCSK9 inhibitors: clinical evidence and implementation
 210. Saleh M, Gabriels J, Chang D, Soo Kim B, Mansoor A, et al. 2020. Effect of Chloroquine, Hydroxychloroquine, and Azithromycin on the Corrected QT Interval in Patients with SARS-CoV-2 Infection. *Circ. Arrhythmia Electrophysiol.*, pp. 496–504
 211. Sampson LJ, Hayabuchi Y, Standen NB, Dart C. 2004. Caveolae localize protein kinase A signaling to arterial ATP-sensitive potassium channels. *Circ. Res.* 95(10):1012–18
 212. Sánchez-Chapula JA, Navarro-Polanco RA, Culberson C, Chen J, Sanguinetti MC. 2002. Molecular determinants of voltage-dependent human ether-a-go-go related gene (HERG) K⁺ channel block. *J. Biol. Chem.* 277(26):23587–95
 213. Sánchez-Chapula JA, Salinas-Stefanon E, Torres-Jácome J, Benavides-Haro DE, Navarro-Polanco RA. 2001. Blockade of Currents by the Antimalarial Drug Chloroquine in Feline Ventricular Myocytes. *J. Pharmacol. Exp. Ther.* 297(1):437 LP – 445
 214. Sanguinetti MC, Jurkiewicz NK. 1990. Two components of cardiac delayed rectifier K⁺ current: Differential sensitivity to block by class III antiarrhythmic agents. *J. Gen. Physiol.* 96(1):195–215
 215. Scharfmann R, Ph D, Bryan J, Aguilar-bryan L, Vaxillaire M, Pharm D. 2006. Activating Mutations in the. *Differences*
 216. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, et al. 2012. Fiji: An open-source platform for biological-image analysis
 217. Schwanstecher M, Löser S, Chudziak F, Bachmann C, Panten U. 1994. Photoaffinity Labeling of the Cerebral Sulfonylurea Receptor Using a Novel Radioiodinated Azidoglibenclamide Analogue. *J. Neurochem.*
 218. Schwanstecher M, Löser S, Chudziak F, Panten U. 1994. Identification of a 38-kDa high affinity sulfonylurea-binding peptide in insulin-secreting cells and cerebral cortex. *J. Biol. Chem.* 269(27):17768–71
 219. Schwanstecher M, Sieverding C, Dörschner H, Gross I, Aguilar-Bryan L, et al. 1998. Potassium channel openers require ATP to bind to and act through sulfonylurea receptors. *EMBO J.* 17(19):5529–35
 220. Sekar S, Gopalakrishnan V, Taghibiglou C. 2017. Sterol regulatory

- element-binding protein 1 inhibitors decrease pancreatic cancer cell viability and proliferation. *Biochem. Biophys. Res. Commun.* 488(1):136–40
221. Sgard F, Faure C, Drieu La Rochelle C, Graham D, O'Connor SE, et al. 2000. Regulation of ATP-sensitive potassium channel mRNA expression in rat kidney following ischemic injury. *Biochem. Biophys. Res. Commun.* 269(2):618–22
 222. Shao W, Espenshade PJ. 2012. Expanding roles for SREBP in metabolism. *Cell Metab.* 16(4):414–19
 223. Shaw RM, Rudy Y. 1997. Electrophysiologic effects of acute myocardial ischemia: a theoretical study of altered cell excitability and action potential duration¹. *Cardiovasc. Res.* 35(2):256–72
 224. Shi NQ, Ye B, Makielski JC. 2005. Function and distribution of the SUR isoforms and splice variants. *J. Mol. Cell. Cardiol.* 39(1):51–60
 225. Shyng S-L, Nichols CG. 1997. Octameric Stoichiometry of the K ATP Channel Complex. *J. Gen. Physiol.* 110:655–64
 226. Shyng SL, Cukras CA, Harwood J, Nichols CG. 2000. Structural determinants of PIP₂ regulation of inward rectifier K(ATP) channels. *J. Gen. Physiol.* 116(5):599–607
 227. Shyng SL, Ferrigni T, Nichols GG. 1997. Control of rectification and gating of cloned K(ATP) channels by the Kir6.2 subunit. *J. Gen. Physiol.* 110(2):141–53
 228. Shyng SL, Nichols CG. 1998. Membrane phospholipid control of nucleotide sensitivity of K(ATP) channels. *Science (80-.).* 282(5391):1138–41
 229. Simard JM, Chen M, Tarasov K V, Bhatta S, Ivanova S, et al. 2006. Newly expressed SUR1-regulated NCCa-ATP channel mediates cerebral edema after ischemic stroke. *Nat. Med.* 12(4):433–40
 230. Singlas E. 1995. Pharmacocinétique clinique de l'azithromycine [Clinical pharmacokinetics of azithromycin]. *Pathol Biol.* 43(6):505–11
 231. Sounderajah V, Ashrafian H, Aggarwal R, De Fauw J, Denniston AK, et al. 2020. Developing specific reporting guidelines for diagnostic accuracy studies assessing AI interventions: The STARD-AI Steering Group. *Nat. Med.* 26(6):807–8
 232. Spruce AE, Standen NB, Stanfield PR. 1985. Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature.* 316(22):

233. Spruce AE, Standen NB, Stanfield PR. 1987. STUDIES OF THE UNITARY PROPERTIES OF ADENOSINE-5'-TRIPHOSPHATE-REGULATED POTASSIUM CHANNELS OF FROG SKELETAL MUSCLE. *J. Physiol.* 382:213–36
234. Standen NB, Quayle JM, Davies NW, Brayden JE, Huang Y, Nelson MT. 1989. Hyperpolarizing vasodilators activate ATP-sensitive K⁺ channels in arterial smooth muscle. *Science* (80-.). 245(4914):177–80
235. Stern MD, Silverman HS, Houser SR, Josephson RA, Capogrossi MC, et al. 1988. Anoxic contractile failure in rat heart myocytes is caused by failure of intracellular calcium release due to alteration of the action potential. *Proc. Natl. Acad. Sci. U. S. A.* 85(18):6954–58
236. Stoller DA, Fahrenbach JP, Chalupsky K, Tan BH, Aggarwal N, et al. 2010. Cardiomyocyte sulfonylurea receptor 2-K ATP channel mediates cardioprotection and ST segment elevation. *Am. J. Physiol. - Hear. Circ. Physiol.* 299(4):1100–1108
237. Sturgess NC, Cook DL, Ashford MLJ, Hales CN. 1985. the Sulphonylurea Receptor May Be an Atp-Sensitive Potassium Channel. *Lancet.* 326(8453):474–75
238. Su F, Shi M, Yan Z, Ou D, Li J, et al. 2012. Simvastatin modulates remodeling of Kv4.3 expression in rat hypertrophied cardiomyocytes. *Int. J. Biol. Sci.* 8(2):236–48
239. Sun X, Nunes SS. 2017. Bioengineering approaches to mature human pluripotent stem cell-derived cardiomyocytes. *Front. Cell Dev. Biol.* 5(MAR):1–8
240. Suzuki M, Li RA, Miki T, Uemura H, Sakamoto N, et al. 2001. Potassium Channels Clarified by Kir6 . 2-Knockout Mice. *Circ. Res.*, pp. 570–77
241. Suzuki M, Sasaki N, Miki T, Sakamoto N, Ohmoto-sekine Y, et al. 2002. Role of sarcolemmal KATP channels in cardioprotection against ischemia/reperfusion injury in mice. *J. Clin. Invest.* 109(4):509–16
242. Swank KA, Wu E, Kortepeter C, McAninch J, Levin RL. 2017. Adverse event detection using the FDA post-marketing drug safety surveillance system: Cardiotoxicity associated with loperamide abuse and misuse. *J. Am. Pharm. Assoc.* 57(2):S63–67
243. Szilvassy Z, Ferdinandy P, Szilvassy J, Nagy I, Karcsu S, et al. 1995. The loss of pacing-induced preconditioning in atherosclerotic rabbits: Role of hypercholesterolaemia. *J. Mol. Cell. Cardiol.* 27(12):2559–69
244. Terrenoire C, Wang K, Chan Tung KW, Chung WK, Pass RH, et al. 2013.

- Induced pluripotent stem cells used to reveal drug actions in a long QT syndrome family with complex genetics. *J. Gen. Physiol.* 141(1):61–72
245. Tisdale JE, Chung MK, Campbell KB, Hammadah M, Joglar JA, et al. 2020. Drug-Induced Arrhythmias: A Scientific Statement from the American Heart Association. *Circulation*, pp. E214–33
 246. Tohyama S, Hattori F, Sano M, Hishiki T, Nagahata Y, et al. 2013. Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell Stem Cell.* 12(1):127–37
 247. Toth JJ, Datta S, Athanikar JN, Freedman LP, Osborne TF. 2004. Selective Coactivator Interactions in Gene Activation by SREBP-1a and -1c. *Mol. Cell. Biol.* 24(18):8288–8300
 248. Traebert M, Dumotier B, Meister L, Hoffmann P, Dominguez-Estevéz M, Suter W. 2004. Inhibition of hERG K⁺ currents by antimalarial drugs in stably transfected HEK293 cells. *Eur. J. Pharmacol.* 484(1):41–48
 249. Trapp S, Proks P, Tucker SJ, Ashcroft FM. 1998. Molecular analysis of ATP-sensitive K channel gating and implications for channel inhibition by ATP. *J. Gen. Physiol.* 112(3):333–49
 250. Tucker SJ, Gribble FM, Proks P, Trapp S, Ryder TJ, et al. 1998. Molecular determinants of K(ATP) channel inhibition by ATP. *EMBO J.* 17(12):3290–96
 251. Tucker SJ, Gribble FM, Zhao C, Trapp S, Ashcroft FM. 1997. Truncation of Kir2.2 produces ATP-sensitive K⁺ channels in the absence of the sulphonylurea receptor. *Nature.* 387(May):179–83
 252. Turer AT, Hill JA. 2010. Pathogenesis of Myocardial Ischemia-Reperfusion Injury and Rationale for Therapy. *Am. J. Cardiol.* 106(3):360–68
 253. Uhde I, Toman A, Gross I, Schwanstecher C, Schwanstecher M. 1999. Identification of the potassium channel opener site on sulfonylurea receptors. *J. Biol. Chem.* 274(40):28079–82
 254. Ungi I, Ungi T, Ruzsa Z, Nagy E, Zimmermann Z, et al. 2005. Hypercholesterolemia attenuates the anti-ischemic effect of preconditioning during coronary angioplasty. *Chest.* 128(3):1623–28
 255. Vaidyanathan R, Markandeya YS, Kamp TJ, Makielski JC, January CT, Eckhardt LL. 2016. IK1-enhanced human-induced pluripotent stem cell-derived cardiomyocytes: An improved cardiomyocyte model to investigate inherited arrhythmia syndromes. *Am. J. Physiol. - Hear. Circ. Physiol.* 310(11):H1611–21

256. Vakkalanka JP, Charlton NP, Holstege CP. 2017. Epidemiologic Trends in Loperamide Abuse and Misuse. *Ann. Emerg. Med.* 69(1):73–78
257. Vandenberg JI, Perry MD, Perrin MJ, Mann SA, Ke Y, Hill AP. 2012. hERG K(+) channels: structure, function, and clinical significance. *Physiol. Rev.* 92(3):1393–1478
258. Varon J, Marik PE. 2000. The diagnosis and management of hypertensive crises. *Chest.* 118(1):214–27
259. VAUGHN P, SOLIK MMK, BAGGA S, PADANILAM BJ. 2016. Electrocardiographic Abnormalities, Malignant Ventricular Arrhythmias, and Cardiomyopathy Associated With Loperamide Abuse. *J. Cardiovasc. Electrophysiol.* 27(10):1230–33
260. Vincent MJ, Bergeron E, Benjannet S, Erickson BR, Rollin PE, et al. 2005. Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. *Viol. J.* 2:1–10
261. Wakatsuki T, Nakaya Y, Inoue I. 1992. Vasopressin modulates K⁺-channel activities of cultured smooth muscle cells from porcine coronary artery. *Am. J. Physiol. - Hear. Circ. Physiol.*
262. Wang M, Cao R, Zhang L, Yang X, Liu J, et al. 2020. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* 30(3):269–71
263. Wang X, Sato R, Brown MS, Hua X, Goldstein JL. 1994. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell.* 77(1):53–62
264. Weiss JN, Venkatesh N, Lamp ST. 1992. ATP-sensitive K⁺ channels and cellular K⁺ loss in hypoxic and ischaemic mammalian ventricle. *J. Physiol.* 447(1):649–73
265. Wojtovich AP, Urciuoli WR, Chatterjee S, Fisher AB, Nehrke K, Brookes PS. 2013. Kir6.2 is not the mitochondrial KATP channel but is required for cardioprotection by ischemic preconditioning. *Am. J. Physiol. - Hear. Circ. Physiol.* 304(11):1439–45
266. Woo SK, Kwon MS, Geng Z, Chen Z, Ivanov A, et al. 2012. Sequential activation of hypoxia-inducible factor 1 and specificity protein 1 is required for hypoxia-induced transcriptional stimulation of Abcc8. *J. Cereb. Blood Flow Metab.* 32(3):525–36
267. Wu PE, Juurlink DN. 2017. Clinical Review: Loperamide Toxicity. *Ann. Emerg. Med.* 70(2):245–52

268. Yang X, Pabon L, Murry CE. 2014. Engineering adolescence: Maturation of human pluripotent stem cell-derived cardiomyocytes. *Circ. Res.* 114(3):511–23
269. Yang YJ, Zhao JL, You SJ, Wu YJ, Jing ZC, et al. 2007. Post-infarction treatment with simvastatin reduces myocardial no-reflow by opening of the KATP channel. *Eur. J. Heart Fail.* 9(1):30–36
270. Yang Z, Prinsen JK, Bersell KR, Shen W, Yermalitskaya L, et al. 2017. Azithromycin Causes a Novel Proarrhythmic Syndrome. *Circ. Arrhythmia Electrophysiol.* 10(4):1–12
271. Yokoyama C, Wang X, Briggs MR, Admon A, Wu J, et al. 1993. SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell.* 75(1):187–97
272. York NW, Parker H, Xie Z, Tyus D, Waheed MA, et al. 2020. Kir6.1- and SUR2-dependent KATP over-activity disrupts intestinal motility in murine models of Cantu Syndrome. *JCI Insight.* 5(23):
273. Yoshida H, Feig JE, Morrissey A, Ghiu IA, Artman M, Coetzee WA. 2004. KATP channels of primary human coronary artery endothelial cells consist of a heteromultimeric complex of Kir6.1, Kir6.2, and SUR2B subunits. *J. Mol. Cell. Cardiol.* 37(4):857–69
274. Yoshida Y, Yamanaka S. 2017. Induced Pluripotent Stem Cells 10 Years Later. *Circ. Res.* 120(12):1958–68
275. Zerangue N, Schwappach B, Yuh NJ, Lily YJ. 1999. A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron.* 22(3):537–48
276. Zhao TC, Cheng G, Zhang LX, Tseng YT, Padbury JF. 2007. Inhibition of histone deacetylases triggers pharmacologic preconditioning effects against myocardial ischemic injury. *Cardiovasc. Res.* 76(3):473–81
277. Zimmermann WH. 2013. Biomechanical regulation of in vitro cardiogenesis for tissue-engineered heart repair. *Stem Cell Res. Ther.* 4(6):
278. Zingman L V, Hodgson DM, Bast PH, Kane GC, Perez-Terzic C, et al. 2002. Kir6.2 is required for adaptation to stress. *Proc. Natl. Acad. Sci. U. S. A.* 99(20):13278–83