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Investigating Novel Compounds in the Treatment of Cardiac Arrhythmia

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INVESTIGATING NOVEL COMPOUNDS IN THE TREATMENT OF CARDIAC ARRHYTHMIA

By

Briana Marie Bohannon

A DISSERTATION

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INVESTIGATING NOVEL COMPOUNDS IN THE TREATMENT OF CARDIAC ARRHYTHMIA

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Cardiac arrhythmia leads to 325,000 cases of sudden cardiac death annually in adults alone (Clinic 2017). Long QT Syndrome (LQTS) is an arrhythmogenic disorder that is caused by mutations (or channelopathies) in voltage-gated ion channels expressed in the cardiomyocyte. Polyunsaturated fatty acids (PUFAs) have emerged as a potential therapeutic for the treatment of LQTS because they modulate the activity of voltage-gated ion channels involved in LQTS pathology. PUFAs are amphipathic molecules with a hydrophilic head group, as well as long, polyunsaturated hydrophobic tail. In this work, we demonstrate that the position of the double bonds in the hydrophobic tail influence the apparent binding affinity of the PUFA to the cardiac Kv7.1/KCNE1 channel complex. We also find that alterations to the hydrophilic head group (i.e. substituting a carboxyl head for a glycine or taurine group) result in a range of Kv7.1/KCNE1 channel activators. Lastly, we find that PUFAs range in their selectivity for different voltage-gated channels expressed in the heart (e.g. Nav1.5, Cav1.2, and Kv7.1/KCNE1). Specifically, PUFA analogues with taurine head groups tend to have broad effects on multiple voltage-gated ion channels including Nav1.5, Cav1.2, and Kv7.1/KCNE1. However, PUFA analogues with glycine head groups tend to have more selective effects on Kv7.1/KCNE1 channels. PUFA analogues with glycine
head groups such as pinoleoyl glycine and DHA-glycine are able to partially restore a prolonged ventricular action potential and prevent arrhythmia in simulated cardiomyocytes. These results suggest a therapeutic role of polyunsaturated fatty acid analogues in the treatment of cardiac arrhythmia.
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LIST OF ABBREVIATIONS

AV – atrioventricular node
CMC – critical micellar concentration
DHA – docosahexaenoic acid
DHP – dihydropyridine
ECG – electrocardiogram
EPA – eicosapentaenoic acid
FA – fatty acid
FFA – free fatty acid
LQTS – Long QT Syndrome
MUFA – monounsaturated fatty acid
N-AT – N-arachidonoyl taurine
PUFA – polyunsaturated fatty acid
SA – sinoatrial node
SFA – saturated fatty acid
TdP – Torsades de Pointes
TTX – tetrodotoxin
CHAPTER 1: Introduction

1.1. Overview of the electrical activity of the heart and Long QT Syndrome.

The human heart operates as a pump, allowing blood to circulate throughout the body (Mohrman and Heller 2010). The normal cardiac cycle depends on precisely timed electrical activity of cardiac muscle cells (cardiomyocytes) (Malanga 2007). The cardiac action potential is the electrical signal that is propagated through the heart, leading ultimately to the coordinated contraction of the heart muscle (Feher 2012) (Figure 1.1). The initiation of the cardiac cycle begins in the sinoatrial (SA) node of the heart, which contains pacemaker cells that allow spontaneous, rhythmic, action potential firing (Feher 2012; Prinzen et al. 2017; Mohrman and Heller 2010). The action potential that is initiated in the SA node travels through the atrial muscle and then reaches the atrioventricular (AV) node (Feher 2012; Prinzen et al. 2017; Mohrman and Heller 2010). From the AV node, the action potential is conducted into the ventricular muscle via Purkinje fibers (Feher 2012; Prinzen et al. 2017; Mohrman and Heller 2010). There is a notable change in the action potential waveform when the action potential reaches the Purkinje fibers and ventricles, including a long plateau phase following the initial depolarization phase (Feher 2012; Prinzen et al. 2017; Mohrman and Heller 2010). During this long plateau phase, calcium enters the cells allowing the synchronized contraction of the ventricular muscle, allowing blood to be pumped from the heart to the rest of the body and circulate systemically (Feher 2012; Prinzen et al. 2017; Mohrman and Heller 2010; Bers and Perez-Reyes 1998). The timing and duration of the cardiac action potential is...
determined by the expression profile of cardiac ion channels that have distinct voltage-dependent and kinetic properties.

Figure 1.1. The cardiac action potential measured in different regions of the heart and the electrocardiogram (ECG). The cardiac action potential measured in the sinoatrial (SA) node, the atrioventricular (AV) node, and in the ventricular muscle as they correspond to the electrocardiogram (ECG). The P wave indicates depolarization of the atrium and aligns with the initiation of the action potential in the SA node. The QRS complex indicates depolarization of the ventricles and aligns with the initiation of the action potential in the ventricular muscle. The T wave indicates repolarization of the ventricles and aligns with the repolarization/termination of the action potential in the ventricular muscle.
The cardiac cycle can be measured using the electrocardiogram (ECG) (Mohrman and Heller 2010; Carroll 2007) (Figure 1.1). In the ECG, the P wave indicates depolarization of the atrial muscle; the QRS complex indicates depolarization of the ventricular muscle; and the T wave indicates repolarization of the ventricular muscle (Mohrman and Heller 2010; Carroll 2007). The QT interval, or the time between depolarization and repolarization of the ventricular muscle, closely corresponds to the action potential duration in the ventricular muscle (Mohrman and Heller 2010; Carroll 2007). The ventricular action potential is divided into four phases: Phases 0 – 3 (Figure 1.2). Each of these phases are mediated by the activation and inactivation of different voltage-dependent currents (Mohrman and Heller 2010). Phase 0 is the point at which the ventricular action potential is initiated and an inward Na\(^+\) current, mediated by Nav1.5, leads to rapid depolarization of the membrane potential (Mohrman and Heller 2010; Carroll 2007; Feher 2012). Phase 1, which appears as a “notch” in the ventricular action potential, occurs as a result of the rapid inactivation of Nav1.5 and also the activation of a transient outward K\(^+\) current (I\(_{\text{T}}\)) (Mohrman and Heller 2010; Carroll 2007; Feher 2012). Phase 2, which appears as a plateau in the membrane potential, is mediated by the influx of Ca\(^{2+}\) ions by the L-type Ca\(^{2+}\) channel, Cav1.2 (Mohrman and Heller 2010; Carroll 2007; Feher 2012). Phase 3, or the repolarization of the ventricular action potential, is initiated when the efflux of K\(^+\) dominates over Ca\(^{2+}\) influx, due to the slow inactivation of Cav1.2 and the activation the delayed rectifier currents, I\(_{\text{Kr}}\) and I\(_{\text{Ks}}\) (Mohrman and Heller 2010; Carroll 2007; Feher 2012; Nerbonne and Kass 2005). The I\(_{\text{Kr}}\) current is the rapid
component of the delayed rectifier $K^+$ current and is comprised of the Kv11.1 channel co-assembled with the KCNE2 auxiliary subunit (Nerbonne and Kass 2005). The $I_{KS}$ current is the slow component of the delayed rectifier $K^+$ current and is mediated by the Kv7.1 channel co-assembled with the KCNE1 auxiliary subunit (Nerbonne and Kass 2005).

Figure 1.2. Phases of the ventricular action potential and underlying channel currents. (Adapted from website image. What-when-how: The Cardiac Ion Channels (Cardiac Arrhythmias) Part 1)). Phases 0-4 of the cardiac ventricular action potential. Phase 0 is mediated by activation of Nav1.5 channels and inward Na$^+$ current. Phase 1 is mediated by the inactivation of Nav1.5 channels. Phase 2 is mediated by activation of Cav1.2 channels and inward Ca$^{2+}$ current. Phase 3 is mediated by the inactivation of Cav1.2 channels and the activation of delayed rectifier potassium channels Kv7.1/KCNE1 ($I_{KS}$ current) and Kv11.1 ($I_{Kr}$ current) and outward K$^+$ current.
Disruption of the cardiac action potential can lead to various forms of cardiac arrhythmia (Schwartz, Crotti, and Insolia 2012; Wu, Ding, and Horie 2016). Long QT Syndrome (LQTS) is an arrhythmogenic disorder that results from the aberrant activity of various cardiac ion channels (Balse and Boycott 2017; Clancy and Kass 2005; Kass and Cabo 2000; Sanguinetti 1999; Sanguinetti et al. 1995; Schwartz, Crotti, and Insolia 2012). LQTS is characterized by a prolongation of the QT interval – the time between depolarization and repolarization of the ventricular muscle – which, in turn, corresponds to a prolongation of the ventricular action potential duration (Alders and Christiaans 2003; Bohnen et al. 2017; Roden 2008; Rossenbacker and Priori 2007; Schwartz, Crotti, and Insolia 2012; Waddell-Smith and Skinner 2016). A prolonged QT interval is diagnosed when it is longer than 440 ms in men and longer than 460 ms in women (Rossenbacker and Priori 2007). There are several different subtypes of LQTS (LQ1-LQT15) which are caused by mutations in different ion channels or other proteins (Alders and Christiaans 2003; Bohnen et al. 2017; Roden 2008; Schwartz, Crotti, and Insolia 2012; Fernandez-Falgueras et al. 2017; Schwartz, Ackerman, and Wilde 2017). LQTS predisposes patients to a particularly dangerous form of cardiac arrhythmia known as Torsades de Pointes (TdP), which is a re-entrant arrhythmia resulting in abnormal T/U waves giving the appearance of a “twisting of the points” (Yap and Cam 2003; Viskin 1999). Torsades de Pointes that is non-terminating can degenerate into ventricular fibrillation and sudden cardiac death (Yap and Cam 2003; Viskin 1999; Fernandez-Falgueras et al. 2017; Schwartz, Ackerman, and Wilde 2017).
Common treatments for LQTS include the prescription of β-blockers and the implantation of a cardioverter defibrillator, both of which act to prevent the likelihood of an arrhythmic event, either by reducing adrenergic tone of the heart or by delivering an electrical impulse to terminate arrhythmia (Cho 2016; Schwartz, Crotti, and Insolia 2012; Waddell-Smith and Skinner 2016). However, these treatments do not target the underlying channelopathies that lead to LQTS.

1.2. Cardiac voltage-gated ion channels.
The voltage-gated Na⁺ channel, Nav1.5, is a TTX insensitive Naᵥ channel that is expressed in cardiac tissue (DeMarco and Clancy 2016). Nav1.5 contains four non-identical linked domains which are called DI-DIV (Catterall 2000; Yu and Catterall 2003; Catterall, Wisedchaisri, and Zheng 2017) (Figure 1.3A). Each of these domains contain 6 transmembrane helices, called S1-S6, which are divided into a voltage-sensing domain and a pore domain (Yu and Catterall 2003; DeMarco and Clancy 2016). The voltage sensing domain is made up of the S1-S4 transmembrane segments (DeMarco and Clancy 2016). The S4 helix contains 4 positively charged amino acid residues (R1-R4; 3 arginines and 1 lysine), allowing it to act as the voltage sensor where it detects and responds to changes in membrane potential (Bezanilla 2008; Moreau et al. 2015). S5-S6 form the pore domain. The Nav1.5 α-subunit can be modulated by being co-assembled with auxiliary β-subunits, including β1 and β3 (Barro-Soria, Liin, and Larsson 2017; Catterall 2000; Yu and Catterall 2003). In the ventricles of the heart, Nav1.5 is assembled with the β3 subunit (Ko et al. 2005). In some cases,
co-expression of Nav1.5 with β-subunits has been shown to alter the pharmacology of Nav1.5 (Xiao et al. 2000). Nav1.5 activates upon membrane depolarization, allowing the influx of Na\(^+\) ions before it quickly inactivates (Armstrong and Bezanilla 1977; Bezanilla and Armstrong 1977; Capes et al. 2013; Chanda and Bezanilla 2002). Work characterizing the contributions of different domains to fast inactivation of Nav channels have shown that the movement of the domain IV voltage sensor is necessary and sufficient to produce fast inactivation (Capes et al. 2013). Gain-of-function mutations of the Nav1.5 channel lead to Long QT Syndrome Type 3 (LQT3) (Callow et al. 2013; Fernandez-Falgueras et al. 2017).

The voltage-gated Ca\(^{2+}\) channel, Cav1.2, is a voltage-activated, L-type Ca\(_v\) channel expressed in cardiac tissue (Hofmann et al. 2014). Cav1.2, like Nav1.5, contains four non-identical linked domains, DI-DIV (Figure 1.3B). Each domain contains S1-S6 helices and the S4 helix behaves as the voltage sensor in each of these domains (Hofmann et al. 2014; Pantazis et al. 2014). The Cav1.2 channel exists as a large macromolecular complex comprising the Cav1.2 α-subunit, as well as β1 and α2δ auxiliary subunits (Rougier and Abriel 2016). These auxiliary subunits, β1 and α2δ alter channel expression and the activation and deactivation kinetics, respectively (Rougier and Abriel 2016; Savalli et al. 2016). Cav1.2 channel is activated upon depolarization and Cav1.2 current is active during the plateau phase of the ventricular action potential, allowing Ca\(^{2+}\) influx that is required for excitation-contraction coupling in the cardiac muscle.
(Bers and Perez-Reyes 1998; Mohrman and Heller 2010). Cav1.2 channels undergo two forms of inactivation: voltage-dependent inactivation and calcium-dependent inactivation, which is a mechanism to restrict excessive calcium influx (Stotz, Jarvis, and Zamponi 2003; Zhang et al. 1994; Abderemane-Ali et al. 2019). Cav1.2 channels are sensitive to dihydropyridines (DHPs), which inhibit the activity of Cav1.2 channels through an allosteric mechanism (Tang et al. 2016). Gain-of-function mutations in Cav1.2 channels lead to Long QT Syndrome Type 8 (LQT8) (Dick et al. 2016; Drum et al. 2014).

The slow component of the delayed rectifier K⁺ current, Iₖₛ current, is mediated by Kv7.1 and the KCNE1 auxiliary β-subunit (Barhanin et al. 1996; Salata et al. 1996; Sanguinetti 1996) (Figure 1.3C). Kv7.1 is a voltage-gated K⁺ channel that is encoded by the KCNQ1 gene and assembles as a tetrameric channel (Smith et al. 2007; Sun and MacKinnon 2017). A single Kv7.1 subunit is made up of 6 transmembrane spanning helices, where S1-S4 make up the voltage sensing domain (VSD), S4 acts as the voltage sensor, and S1-S6 make up the pore domain (PD) (Peroz et al. 2008; Smith et al. 2007; Sun and MacKinnon 2017). 4 Kv7.1 subunits form a homotetrameric channel which is activated by depolarization and allows the selective efflux of K⁺ ions (Smith et al. 2007; Sun and MacKinnon 2017). KCNE1 (also called minK) is a single transmembrane spanning protein that is necessary to recapitulate physiological Iₖₛ current. KCNE1 is suggested to localize in the lipophilic cleft between adjacent voltage sensing domains of Kv7.1 (Chung et al. 2009; Murray et al. 2016; Nakajo et al.
In this position, KCNE1 is able to alter the voltage-dependence of Kv7.1, causing it to open at more positive potentials (Gofman 2012; Osteen et al. 2010; Barro-Soria et al. 2014). In addition, KCNE1 slows the activation kinetics of Kv7.1 and also increases the single channel conductance (Yang and Sigworth 1998; Panaghie, Tai, and Abbott 2006). Loss-of-function mutations of the Kv7.1/KCNE1 (I_{Ks}) channel lead to Long QT Syndrome Type 1 (LQT1), which is the most common form of LQTS (Eldstrom et al. 2010; Wu, Ding, and Horie 2016; Crotti et al. 2007; Huang et al. 2018; Ma et al. 2015).
Figure 1.3. Membrane topology and auxiliary subunits of cardiac voltage-gated ion channels. **A)** Topology of the cardiac Nav1.5 α-subunit (light blue) and auxiliary β1 subunit (green). **B)** Topology of the cardiac Cav1.2 α-subunit (light gray) and auxiliary β3 (mint green) and α2δ (yellow and lime green) subunits. **C)** Topology of the cardiac Kv7.1 α-subunit (cyan) and auxiliary β-subunit KCNE1 (dark gray).
1.3. Polyunsaturated fatty acids (PUFAs).

Polyunsaturated fatty acids (PUFAs) are amphipathic molecules that have both a hydrophilic head group and a hydrophobic tail group (Benatti et al. 2004; Foundation 1992) (Figure 1.4A). The hydrophobic tail of the PUFA molecule can range from 14-22 carbons in length and contains 2 or more double bonds in order to be classified as polyunsaturated (Benatti et al. 2004; Foundation 1992). In naturally occurring PUFAs, the hydrophilic head group is a –COOH, or carboxyl, group (Benatti et al. 2004; Foundation 1992). This carboxyl group has the potential to be negatively charged when the PUFA head group is deprotonated (Benatti et al. 2004; Foundation 1992). Polyunsaturated fatty acids (PUFAs), for example, docosahexaenoic acid (DHA) has well-documented roles in the development of the nervous system and is also suggested to reduce the risk of coronary artery disease (Horrocks and Yeo 1999) (Figure 1.4A). PUFAs became of clinical interest historically when researchers found that there was an Inuit population with a very low incidence of cardiovascular disease (Horrocks and Yeo 1999; Bang, Dyerberg, and Hjoorne 1976). This population consumed a diet that was enriched in fish oil and therefore had elevated plasma concentration of free fatty acids (FFAs) (Bang, Dyerberg, and Hjoorne 1976). ω-3 fatty acids, which are found in fish oils, then became of interest to researchers as potential therapeutic compounds for cardiovascular disease and cardiac arrhythmia. In animal models, such as canine and rabbit models, PUFAs such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were shown to be antiarrhythmic because of their ability to terminate arrhythmic activity (Kang
and Leaf 2000; Pound, Kang, and Leaf 2001). Further work demonstrated that PUFAs have these anti-arrhythmic effects because they modulate the activity of voltage-gated ion channels expressed in the heart, particularly voltage-gated Na\(^+\) (Nav) and Ca\(^{2+}\) channels (Cav) (Elinder and Liin 2017; Kang and Leaf 1996; Xiao et al. 1997; Xiao et al. 1995; Xiao et al. 2001). In the case of Nav and Cav channels, PUFAs reduce Na\(^+\) and Ca\(^{2+}\) currents, allowing them to behave anti-arrhythmically (Elinder and Liin 2017; Kang and Leaf 1996; Xiao et al. 1997; Xiao et al. 1995).

Our group has also demonstrated that PUFAs can promote the activation of voltage-gated K\(^+\) channels, including Kv7.1 and Kv7.1/KCNE1 (Figure 1.4C-D). PUFAs such as DHA promote the activation of voltage-gated K\(^+\) channels such as Shaker and Kv7.1 by causing a left-shift in the voltage-dependence of channel activation, meaning that these channels are activating at more negative potentials and the PUFA is acting to stabilize the open state of the channel (Figure 1.4C). PUFA-induced activation of K\(^+\) channels such as Shaker and Kv7.1 occurs through the lipoelectric hypothesis (Borjesson, Hammarstrom, and Elinder 2008; Yazdi et al. 2016; Kang and Leaf 1996). The lipoelectric hypothesis states that the PUFA molecule: 1) integrates into the membrane via its hydrophobic tail and 2) electrostatically attracts the positively charged residues of S4 by its negatively charged head group (Borjesson, Hammarstrom, and Elinder 2008). This electrostatic interaction promotes the upward movement of the voltage-sensor which results in a conformational change in the channel protein,
opening the channel (Borjesson and Elinder 2011; Borjesson, Hammarstrom, and Elinder 2008). The lipoelectric hypothesis also suggests that a negatively charged head group, a long hydrocarbon tail, and at least two double bonds in the PUFA tail are necessary to promote activation of Kv channels (Borjesson, Hammarstrom, and Elinder 2008; Liin et al. 2015). Unsaturated and monounsaturated fatty acids do not produce a left-shift in the voltage dependence of activation in Kv channels (Borjesson, Hammarstrom, and Elinder 2008; Liin et al. 2015).

Figure 1.4. X-ray crystal structure of the Kv7.1 α-subunit with putative PUFA binding sites. X-ray crystal structure of the Kv7.1 α-subunit (PDB 5VMS; Sun and MacKinnon, 2018, Cell) from the side view (top) and the top-down view (bottom). Transmembrane α-helical segments S1-S6 are labeled. PUFA binding site R231 in the S4 segment (blue labeled amino acid) and K326 in the S6 segment (red labeled amino acid) are indicated in each subunit.
Interestingly, co-expression of the KCNE1 subunit with Kv7.1 removes the activation induced by PUFAs such as DHA (Liin et al. 2015). However, the voltage-shifting effect of DHA on Kv7.1 can be recovered in Kv7.1/KCNE1 if experiments are performed at pH 9, which creates a pH environment that favors deprotonation of the PUFA head group (Liin et al. 2015). Interestingly, the association of KCNE1 with the Kv7.1 subunit alters the local pH near the binding site of the PUFA, altering the pKa of the PUFA molecule when bound to Kv7.1/KCNE1. KCNE1 does this by altering the conformation of the Kv7.1 turret region, which is made up by the S5-S6 linker (Larsson, Larsson, and Liin 2018). In the S5-S6 linker, there are negatively charged acidic amino acid residues (namely E290) which are moved closer to the binding site of the PUFA when KCNE1 is present (Larsson, Larsson, and Liin 2018). The negatively charged turret residues alter the pKa of the PUFA head group, resulting in the protonation of the head group, such that the PUFA head group will be uncharged at physiological pH. The effects of KCNE1 on the PUFA head group can be circumnavigated by using modified PUFAs (or PUFA analogues) that have a different functional group on the PUFA head that have a lower pKa value.

Examples of PUFA analogues that have a lower pKa head group are DHA-glycine and N-arachidonoyl taurine (N-AT) (Figure 1.4B). DHA-glycine and N-AT have both been demonstrated to promote the activation of Kv7.1/KCNE1 at physiological pH (7.5) by left-shifting the voltage-dependence of activation (Liin et al. 2015; Liin et al. 2016). Two other PUFA analogues that contain glycine and
taurine head groups are linoleoyl glycine (lin-glycine) and linoleoyl-taurine (lin-taurine). When the effects of lin-glycine and lin-taurine on I$_{Ks}$ current have been measured, they increase I$_{Ks}$ current in a dose dependent manner with Hill coefficients of $n = 2.7$ and $n = 1.5$, respectively. This suggests that PUFA analogues exhibit some positive cooperativity in binding to the I$_{Ks}$ channel. PUFA analogues have also been evaluated for their potential to treat LQTS and cardiac arrhythmia. N-AT has been shown in previous work to rescue the wild type activity of several diverse LQTS-causing mutations in Kv7.1 (Liin et al. 2016). In addition, when applied to neonatal rat cardiomyocytes, DHA-glycine application has been shown to restore normal action potential firing following drug-induced arrhythmia cause by the application of chromanol (Liin et al. 2015).

In recent work, an PUFA analogues have been shown to participate in an additional electrostatic effect on the pore of the Kv7.1/KCNE1 channel which is measured as an increase in the maximal conductance ($G_{\text{max}}$) (Liin, Yazdi, et al. 2018) (Figure 1.4D). This dual effect on Kv7.1/KCNE1 activation occurs by a positively charged amino acid residues, K326, in the S6 segment of Kv7.1 (Liin, Yazdi, et al. 2018). The negatively charged head group of a PUFA analogue participates in an electrostatic interaction with the positively charged lysine to increase the $G_{\text{max}}$, and this electrostatic effect can be eliminated if K326 is mutated (Liin, Yazdi, et al. 2018). Additionally, this $G_{\text{max}}$ effect is independent of the electrostatic effect on the S4 segment of Kv7.1/KCNE1 (Liin, Yazdi, et al. 2018). Mutations that remove the left-shifting effect of PUFA analogues do not
disrupt the G_{max} effect and mutations that remove the G_{max} effect do not disrupt the left-shifting effect of PUFA analogues (Liin, Yazdi, et al. 2018). These data further suggest that modulation of the cardiac I_{Ks} channel by PUFA analogues is electrostatic in nature.

Figure 1.5. Polyunsaturated fatty acid (PUFA) structure and the lipoelectric hypothesis. A-B) Structure of A) the PUFA docosahexaenoic acid (DHA) and B) the PUFA analogue N-arachidonoyl taurine (N-AT). C) Electrostatic interaction between the negatively charged PUFA head group (yellow) and positive charges in the voltage sensor segment (green) in Kv channels. D) Electrostatic interaction between the negatively charged PUFA head group (yellow) and positive charges (K326) in the Kv channel pore.
The work described here provides a comprehensive analysis of how the structure of PUFAs and PUFA analogues relate to activation of the cardiac Kv7.1/KCNE1 (I_{Ks}) channel. In addition, we have demonstrated that PUFAs and PUFA analogues vary in their channel selectivity and that they modulate the activity of different voltage-gated ion channels through different mechanisms.
CHAPTER 2. \( \omega-6 \) and \( \omega-9 \) polyunsaturated fatty acids with double bonds near the carboxyl head have the highest affinity and largest effects on the cardiac \( I_{KS} \) potassium channel

**Background**

Excitable tissues, such as brain, heart, and muscle, express a wide variety of voltage-gated ion channels that play a critical role in the firing and the shaping of action potentials (Hille 2001). Voltage-gated \( K^+ \) channels (Kv channels) comprise an important family of channels that are involved in the repolarization phase of the cardiac action potential (Deal, England, and Tamkun 1996; Salata et al. 1996; Lei and Brown 1996; Li et al. 1996; Veldkamp et al. 1995; Noble and Tsien 1969a; Noble and Tsien 1969b). The Kv7.1/KCNE1 macromolecular complex, here forward referred to as the \( I_{KS} \) channel (a slow delayed rectifier Kv channel) is important for the repolarization of the cardiac ventricular action potential. There are a variety of mutations in both the Kv7.1 and KCNE1 subunits that result in a LQTS phenotype and these mutations have been demonstrated to affect an array of processes including voltage sensing, channel opening/closing, and channel trafficking to the membrane (Alders and Christiaans 2003).

Polyunsaturated fatty acids (PUFAs) are naturally occurring, amphipathic molecules that contain a hydrophilic, carboxyl head and hydrophobic

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1 Bohannon, BM., Perez, ME., Liin, SI., and Larsson, HP. (2019) \( \omega-6 \) and \( \omega-9 \) polyunsaturated fatty acids with double bonds near the carboxyl head have the highest affinity and largest effects on the cardiac \( I_{KS} \) potassium channel. *Acta Physiologica*. DOI: 10.1111/alpha.13186
hydrocarbon tail (Benatti et al. 2004). Early evidence from animal models suggested that PUFAs modulate the activity of voltage-gated Na\(^+\) and Ca\(^{2+}\) channels and therefore could act anti-arrhythmically at the ion channel level (Kang and Leaf 1996, 2000; Xiao et al. 1995; Xiao et al. 2001; Xiao et al. 1997). In our lab, we have investigated the potential therapeutic effect of PUFAs on the \(I_{Ks}\) channel and our data strongly suggests that modified PUFAs affect the cardiac \(I_{Ks}\) current by shifting the voltage-dependence of activation to more negative voltages (Liin et al. 2015). PUFAs have also been shown to reverse the loss-of-function of some LQT1 mutants in \(I_{Ks}\) channels expressed in *Xenopus laevis* oocytes and also to reverse drug-induced arrhythmia in cultured cardiomyocytes and a drug-induced prolongation of the QT interval in guinea pig hearts (Liin et al. 2015; Liin et al. 2016). However, the features of these PUFAs that are necessary for the effects on the \(I_{Ks}\) channel are not fully understood. This study investigates the properties of the PUFA tail with the goal of determining what features of the PUFA tail are important for the activating effects of PUFAs on the cardiac \(I_{Ks}\) channel.

Previous data from our lab has shown that the most common PUFA in fish oil, docosahexaenoic acid (DHA), shifts the voltage dependence of activation (\(V_{0.5}\)) of the Kv7.1 channel to more negative voltages by an electrostatic interaction between the negatively charged DHA head group and the positively charged voltage sensor of Kv7.1 (Liin et al. 2015), a mechanism demonstrated in Shaker \(K^+\) channels (Borjesson, Hammarstrom, and Elinder 2008; Borjesson and Elinder
However, when DHA is applied to the physiological $I_{Ks}$ channel (co-expression of Kv7.1 and KCNE1), there is no effect on the $V_{0.5}$. Interestingly, the left-shifting effect of DHA could be restored in the $I_{Ks}$ channel by conducting experiments at pH 9 (rather than pH 7.5) (Liin et al. 2015). We have further shown that the presence of KCNE1 decreases the local pH near the DHA head group, so that DHA is less likely to be negatively charged in the presence of KCNE1 at physiological pH (Liin et al. 2015). By increasing the pH of the extracellular solution to pH = 9, we deprotonate the DHA head group and restore its negative charge, thereby restoring the left-shifting effects of DHA on the $I_{Ks}$ channel (Liin et al. 2015). We have also shown that PUFAs with a lower pKa, such as N-arachidonoyl taurine (N-AT) that remains negatively charged when bound to $I_{Ks}$ channels at pH 7.5, retain the ability to shift the voltage-dependence of Kv7.1 even when it is co-expressed together with KCNE1 (Liin et al. 2016; Liin et al. 2015). While our previous data show the importance of the PUFA head group charge for shifting the $V_{0.5}$ of the $I_{Ks}$ channel, little is known about the importance of PUFA tail properties for the affinity of PUFAs and the effects of PUFAs on the $I_{Ks}$ channel. Due to the limited number of commercially available PUFAs with a permanently negatively-charged head group (such as N-AT), we here tested the importance of the tail properties in PUFAs with a carboxyl head group at pH 9.0 to maintain the negative charge on the head group even in the presence of KCNE1.
Here, we report that specific properties of the hydrocarbon tail are important for the effects of PUFAs on the $I_{Ks}$ current and the apparent binding affinity of PUFAs to the $I_{Ks}$ channel. The length and number of double bonds in the PUFA tail do not strongly correlate with the effects of PUFAs on $I_{Ks}$ current or the binding affinity of PUFAs to the $I_{Ks}$ channel. However, we find that the position of the first double bond strongly correlates with both the effect of PUFAs on $I_{Ks}$ current and the apparent binding affinity of PUFAs for the $I_{Ks}$ channel. PUFAs that have their first double bond close to the hydrophilic head group consistently demonstrate a larger effect on the $I_{Ks}$ current and a higher apparent affinity for the $I_{Ks}$ channel than PUFAs that have their first double bond further away from the PUFA head. In addition, PUFAs with a double bond close to the end of the PUFA tail ($\omega$-3 PUFAs) are less effective compared to PUFAs with a double bond further away from the end of the PUFA tail ($\omega$-6 and $\omega$-9 PUFAs).

**Results**

To determine the effect of a specific PUFA (for example, eicosatrienoic acid 5,8,11), we measured its effects on the current vs. voltage relationship of the $I_{Ks}$ channel (Fig. 1A-C). The tail currents were measured after different activation voltages to generate the G-V curve from which we can determine both the shift in the $V_{0.5}$ and the increase in $G_{\text{max}}$ (Fig.1C) once the current has reached saturation (Fig. 1D). We also measured the relative increase in $K^+$ current ($I/I_0$) at 0 mV (Fig. 1C), a voltage close to the systolic plateau during a cardiac action potential. Both a shift in the $V_{0.5}$ and an increase in the $G_{\text{max}}$ would increase the
currents at 0 mV, so by measuring the currents at 0 mV we get a rough estimate of the total expected physiologically-relevant, PUFA induced change in current during a ventricular action potential (O’Hara et al. 2011). Lastly, we determine $K_m$ values for $I/I_0$, $\Delta V_{0.5}$, and $G_{\text{max}}$ to understand how different properties (i.e. different tail length, double bond number, and bond position) affect the apparent binding affinity of the PUFAs to the $I_{Ks}$ channel (Fig.1E). The apparent binding affinity reported throughout the text is the $K_m$ corresponding to the increase in $I/I_0$, as the apparent affinities of $I/I_0$, $\Delta V_{0.5}$, and $G_{\text{max}}$ are comparable.
2.1. Increasing the length of the PUFA tail does not increase the effects on the $I_{Ks}$ current.

We first investigate the role of the carbon tail length by applying PUFAs of varying length to *Xenopus* oocytes expressing the wild type human $I_{Ks}$ channel. 16 PUFAs were tested that varied in total carbon tail length while maintaining the same (carboxyl) head group (Sup. Fig. 1; Table 1). The recordings were done in solutions at pH 9.0 to ensure a negatively charged head group (Liin et al. 2015). To make it easier to follow the discussion of the different PUFAs, we will refer to PUFAs using a shorthand nomenclature throughout the remainder of this paper: Carbon length: number of double bondsΔposition of double bonds. For example, the tail of docosahexaenoic acid (DHA) has 22 carbons and six double bonds at positions 4,7,10,13,16,19 where the numbering beginning at the carbonyl carbon of the PUFA head group. We will therefore call DHA 22:6Δ4,7,10,13,16,19.
If the length of the carbon tail is important for its effect on the $I_{KS}$ channel, we would expect that changing the length of the carbon tail would produce a subsequent change in $K^+$ current amplitude. However, this is not obvious from the data. As an example, we compare four PUFAs that vary in the length of their carbon tail, but all have a carboxyl head group and have three double bonds in the same position from the end of the tail (22:3Δ13,16,19, 20:3Δ11,14,17, 18:3Δ9,12,15, and 16:3Δ7,10,13). The PUFA with the longest carbon tail (22:3Δ13,16,19) does not increase the maximum $I/I_0$ (0.7 ± 0.01) (Fig. 2A), produces no change in the $G_{max}$ (1.0 ± 0.1) (Sup. Fig. 2), and also produces no left-shift of the $V_{0.5}$ ($\Delta V_{0.5} = 1.7 \pm 0.3$ mV) (Fig. 2B). Removing two carbons from
the tail (20:3Δ11,14,17) increases the maximum $\frac{I}{I_0}$ (1.6 ± 0.1) (Fig. 2A), but does not produce a change in the $G_{\text{max}}$ (1.0 ± 0.1) (Sup. Fig. 2) and produces a small shift in the $V_{0.5}$ ($\Delta V_{0.5} = -4.4 ± 1.3 \text{ mV}$) (Fig. 2B). However, shortening of the PUFA by two additional carbons (18:3Δ9,12,15) produces a three-fold increase in maximum $\frac{I}{I_0}$ (3.3 ± 0.004) (Fig. 2A), slightly increases the $G_{\text{max}}$ (1.6 ± 0.3) (Sup. Fig. 2), and drastically increases the left-shift of the $V_{0.5}$ ($\Delta V_{0.5} = -21.9 ± 0.5 \text{ mV}$) (Fig. 2B). In contrast, further shortening of the PUFA by two more carbons (16:3Δ7,10,13), though it still produces a two-fold increase in maximum $\frac{I}{I_0}$ (2.1 ± 0.02) (Fig. 2A), decreases the left-shift of the $V_{0.5}$ ($\Delta V_{0.5} = -13.4 ± 0.6 \text{ mV}$) (Fig. 2B) and the change in the $G_{\text{max}}$ (0.9 ± 0.2) (Sup. Fig. 2). Overall, when we compare the effects of all 16 PUFAs tested, there is no correlation between the length of the carbon tail and $\frac{I}{I_0}$ (Slope = 0.14 ± 0.12; $R^2 = 0.09$; $p = 0.26$) (Fig. 2C). There is no correlation between the length of the carbon tail and the left shift of the $V_{0.5}$ (Slope = -0.61 ± 1.7; $R^2 = 0.01$; $p = 0.72$) (Fig. 2D). There is a weak correlation between the length of the carbon tail and the maximal conductance ($G_{\text{max}}$) (Slope = 0.08 ± 0.04; $R^2 = 0.25$; $p = 0.05$) (Sup. Fig. 2B). Lastly, there is no correlation between the length of the carbon tail and the apparent binding affinity for $\frac{I}{I_0}$ ($K_m$) (Slope = -0.76 ± 1.3; $R^2 = 0.03$; $p = 0.56$) (Fig. 2E).
Figure 2.3. No role of carbon tail length on PUFA effect and affinity. A) Dose dependent I/I₀ increase in the presence of PUFAAs with varying carbon tail lengths. B) Dose dependent ΔV₀.₅. C-E) Correlation between carbon tail length and (C) I/I₀ (Slope = 0.14 ± 0.12; R² = 0.09; p = 0.26), (D) ΔV₀.₅ (Slope = -0.61 ± 1.7; R² = 0.01; p = 0.72), and (E) K_m of I/I₀ (Slope = -0.76 ± 1.3; R² = 0.03; p = 0.56).
2.2. A greater number of double bonds in the PUFA tail does not increase the effects on $I_{Ks}$ current.

To determine whether the number of double bonds in the tail is important for the effects of PUFAs on the $I_{Ks}$ channel, we look next at PUFAs with different number of double bonds while the head group and the tail length are constant. We first compare two PUFAs (22:5$\Delta$4,7,10,13,16 versus 22:3$\Delta$13,16,19) that both have 22 carbons, but either have 5 or 3 double bonds in the tail. Decreasing the number of double bonds from 5 to 3 bonds (22:5$\Delta$4,7,10,13,16 versus 22:3$\Delta$13,16,19) decreases the $I/I_0$ (3.7 ± 0.02 to 0.7 ± 0.01) (Fig. 3A), removes the increase in $G_{max}$ (1.3 ± 0.02 to 1.0 ± 0.1) (Sup. Fig. 2), and removes the left-shift of the $V_{0.5}$ ($\Delta V_{0.5} = -44.4 \pm 10$ mV to 1.7 ± 0.3 mV) (Fig. 3B). In contrast, reducing the number of double bonds in PUFAs with 20-carbon tails (20:5$\Delta$5,8,11,14,17 versus 20:3$\Delta$5,8,11) increases the maximum $I/I_0$ (2.4 ± 0.05 to 4.0 ± 0.23) (Fig. 3A), slightly increases the $G_{max}$ (1.0 ± 0.1 to 1.3 ± 0.01) (Sup. Fig. 2), and increases the left-shift of the $V_{0.5}$ by a factor of 2 ($\Delta V_{0.5} = -25 \pm 4.6$ mV to -44.7 ± 4.3 mV) (Fig. 3B). Overall, when we compare the effects of all 16 PUFAs tested, there is no correlation between the number of double bonds in the tail and $I/I_0$ (Slope = 0.16 ± 0.29; $R^2 = 0.02; p = 0.59$) (Fig. 3C) There is also no correlation between the number of double bonds and the shift in $V_{0.5}$ (Slope = 0.14 ± 3.8; $R^2 = 0.0001; p = 0.97$) (Fig. 3D), the $G_{max}$ (Sup. Fig. 2C), or the apparent binding affinity of $I/I_0$ ($K_m$) (Slope = -4.2 ± 2.0; $R^2 = 0.28; p = 0.06$) (Fig. 3E).
Figure 2.4. No role of the number of double bonds on PUFA effect and affinity. A) Dose dependent $I/I_0$ increase in the presence of PUFAs with varying number of double bonds. B) Dose dependent changes in $\Delta V_{0.5}$. C-E) Correlation between the number of double bonds and (C) $I/I_0$ (Slope = 0.16 ± 0.29; $R^2$ = 0.02; $p = 0.59$), (D) $\Delta V_{0.5}$ (Slope = 0.14 ± 3.8; $R^2$ = 0.0001; $p = 0.97$), and (E) $K_m$ of $I/I_0$ (Slope = -4.2 ± 2.0; $R^2$ = 0.28; $p = 0.06$).
2.3. Having the first double bonds closer to head group increases apparent affinity and effect of PUFA on \( I_{Ks} \) current.

Because neither the tail length nor the number of double bonds in the tail correlate very strongly with the PUFA-induced effects on the \( I_{Ks} \) channel, we investigated whether the positions of the double bonds are important for the effects of PUFAs on the \( I_{Ks} \) channel. We first compare the compounds 22:6Δ4,7,10,13,16,19 and 22:3Δ13,16,19 on the \( I_{Ks} \) channel (Fig. 4A-B).

Application of 22:6Δ4,7,10,13,16,19 produces a dose dependent increase in maximum \( I/I_0 \) (3.2 ± 0.1) (Fig. 4E), produces an increase in the \( G_{max} \) (1.5 ± 0.2) (Sup. Fig. 2), and produces a significant shift in the \( V_{0.5} \) (Δ\( V_{0.5} = -20.2 ± 0.1 \, \text{mV} \)) (Fig. 4F). However, when the first three double bonds are removed (22:3Δ13,16,19), there is a decrease in maximum \( I/I_0 \) (0.7 ± 0.01) (Fig. 4E), no change in the \( G_{max} \) (1.0 ± 0.1) (Sup. Fig. 2), and the left-shifting effect on the \( V_{0.5} \) is abolished (Δ\( V_{0.5} = 1.7 ± 0.3 \, \text{mV} \)) (Fig. 4F). This data show that some, or all, of the three initial double bonds in the 22:6Δ4,7,10,13,16,19 tail are necessary for its effects on the \( I_{Ks} \) channel. To determine whether reintroducing double bonds closer to the PUFA head improves the effects of 22:3Δ13,16,19, we test 22:4Δ7,10,13,16 and 22:4Δ4,10,13,16 (Fig. 4C-D). 22:4Δ7,10,13,16 application compared to 22:4Δ4,10,13,16 produces a smaller increase in \( I/I_0 \) (3.3 ± 0.01 and 5.3 ± 0.09, respectively) (Fig. 4G), a smaller increase in the \( G_{max} \) (1.5 ± 0.03 and 2.4 ± 0.03, respectively) (Sup. Fig. 2), and produces a marginally smaller shift in the \( V_{0.5} \) (Δ\( V_{0.5} = -14.9 ± 2.2 \, \text{mV} \) and -18.2 ± 0.5 \, \text{mV} \), respectively) (Fig. 4H). In addition, positioning the first double bond closer to the head group increased the
apparent affinity for the $I_{\text{Ks}}$ channel, as 22:4Δ4,10,13,16 had a $K_m = 6.3 \pm 0.3$ μM, whereas 22:4Δ7,10,13,16 had a $K_m = 11.6 \pm 0.1$ μM (for $I/I_0$) ($t$ statistic = 32.2; degrees of freedom = 4; $p < 0.0005$). This suggests that the location of the first double bond close to the hydrophilic head group of the PUFA is important for improving the effects on and the apparent affinity for the $I_{\text{Ks}}$ channel.
Next, we test three 20-carbon PUFAs (20:3Δ5,8,11, 20:3Δ8,11,14, and 20:3Δ11,14,17) that all contain the same number of double bonds, but the double bonds start at different distances from the carboxyl head group (Fig. 5A-C). 20:3Δ5,8,11, which has its first double bond closest in proximity to the head group, produced the largest increase in maximum I/I₀ (4.0 ± 0.2) (Fig. 5D). 20:3Δ8,11,14, in which the double bonds are shifted further away from the head, increased the maximum I/I₀ less than 20:3Δ5,8,11 (2.5 ± 0.04) (Fig. 5D). 20:3Δ11,14,17, in which the double bonds are located furthest away from the PUFA head, produced the smallest increase in maximum I/I₀ (1.6 ± 0.1) (Fig. 5D).

20:3Δ5,8,11 also slightly increased the Gₘₐₓ of the Iₖₛ channel compared to 20:3Δ8,11,14 and 20:3Δ11,14,17 (1.3 ± 0.01, 1.0 ± 0.2, and 1.0 ± 0.1, respectively) (Fig. 5E). 20:3Δ5,8,11 and 20:3Δ8,11,14 both produce a larger left-shift the V₀.₅ (ΔV₀.₅ = -44.7 ± 4.3 mV and -42.5 ± 1.9 mV, respectively) compared to 20:3Δ11,14,17 (-4.4 ± 1.3 mV) (Fig. 5F). 20:3Δ5,8,11 and 20:3Δ8,11,14 bound to the Iₖₛ channel with comparable Kₘ for I/I₀ (9.2 ± 1.4 μM and 14.4 ± 2.5 μM, respectively) and exhibited better apparent affinity for I/I₀ compared to 20:3Δ11,14,17 (35.2 ± 0.1 μM) (Fig. 5G). The effects and apparent affinity of 20:3Δ5,8,11 compared to those of 20:3Δ8,11,14 and 20:3Δ11,14,17 suggest that the location of the first double bond close to the hydrophilic head group of the
PUFA is important for improving the effects on and the apparent affinity for the $I_{Ks}$ channel.
To further test the importance of the location of double bonds relative to the head group, we compared three 18-carbon PUFAs (18:3Δ5,9,12, 18:3Δ6,9,12, and 18:3Δ9,12,15) that all contain the same number of double bonds, but with the double bonds at different locations (Fig. 6A-C). 18:3Δ5,9,12 and 18:3Δ9,12,15 both produce the largest increases in maximum \( I/I_0 \) when compared to 18:3Δ6,9,12 (2.9 ± 0.07 and 3.3 ± 0.004 compared to 2.2 ± 0.06) (Fig. 6D). Both 18:3Δ5,9,12 and 18:3Δ9,12,15 increased the \( G_{\text{max}} \) slightly (1.1 ± 0.01 and 1.6 ± 0.3, respectively) (Sup. Fig. 2), however 18:3Δ6,9,12 did not increase \( G_{\text{max}} \) (0.9 ± 0.1) (Sup. Fig. 2). 18:3Δ5,9,12 application resulted in the greatest left-shift in the \( V_{0.5} \) of \( I_{Ks} \) channel activation, shifting the \( V_{0.5} \) by -38 ± 2.7 mV (Fig. 6E). In contrast, 18:3Δ9,12,15 and 18:3Δ6,9,12 both produced similar left-shifts of the \( V_{0.5} \) (\( \Delta V_{0.5} = -21.9 \pm 0.5 \) mV and -22.3 ± 0.5 mV, respectively) (Fig. 6E). In addition, 18:3Δ5,9,12 has the highest apparent affinity (6.7 ± 0.4 μM), whereas 18:3Δ6,9,12 and 18:3Δ9,12,15 exhibited lower apparent binding affinity for \( I/I_0 \) (10.0 ± 1.0 μM and 18.7 ± 0.1 μM, respectively) (Fig. 6F). These data further suggest that the location of the first double bond close to the hydrophilic head group of the PUFA is important for improving the effects on and apparent affinity for the \( I_{Ks} \) channel.
Figure 2.7. Having the first double bond closer to PUFA head in linolenic acid analogues improves PUFA effects on I/I₀ and ΔV₀.5. A-C) K⁺ current in 0 μM (Left) and 70 μM (Right) for (A) 18:3Δ9,12,15, (B) 18:3Δ6,9,12, and (C) 18:3Δ5,9,12. D-E) Comparison of dose dependent (D) I/I₀ increase and (E) ΔV₀.5 between 18:3Δ9,12,15, 18:3Δ6,9,12, and 18:3Δ5,9,12. F) Correlation between the position of the first double bond and Kₘ of I/I₀ (Slope = 3.0 ± 0.08; Adjusted R² = 0.99; p = 0.02).
To corroborate our conclusion for the importance of the position of the first double bond, we compared three PUFAs (20:3Δ5,8,11, and 22:5Δ4,7,10,13,16, and 18:3Δ5,9,12) (Fig. 7A-C). These three PUFAs all have the first double bond at positions 4 or 5 but they have different carbon tails lengths, a different number of double bonds, and different positions of the other double bonds. The prediction is that PUFAs with the first double bond close to the head group will be similarly effective compounds and have similar apparent affinity for the $I_{Ks}$ channel. 18:3Δ5,9,12, 22:5Δ4,7,10,13,16, and 20:3Δ5,8,11 all increase the maximum $I/I_0$ (2.9 ± 0.07, 3.7 ± 0.02, and 4.0 ± 0.23, respectively) (Fig. 7D), and $G_{max}$ (1.1 ± 0.01, 1.3 ± 0.02, and 1.3 ± 0.01, respectively) (Fig. 7E), and produce a similar shift in the $V_{0.5}$ of the $I_{Ks}$ channel ($ΔV_{0.5}$ = -38 ± 2.7 mV, -44.4 ± 10 mV, and -44.7 ± 4.3 mV, respectively) (Fig. 7F). 18:3Δ5,9,12, 22:5Δ4,7,10,13,16, and 20:3Δ5,8,11, though they are significantly different in their apparent binding affinities for $I/I_0$, ($K_m$ = 6.7 ± 0.4 μM versus 3.7 ± 0.1 and 9.2 ± 1.4 μM, respectively; One-way ANOVA F statistic = 56.3; p < 0.001) are all among the PUFAs tested here that have the highest apparent binding affinity for the $I_{Ks}$ channel. When we compared the effects of all 16 PUFAs tested, there was a significant correlation between the position of the first double bond and $I/I_0$ (Slope = -0.24 ± 0.1; $R^2 = 0.27$; p = 0.04) (Fig. 7G). There is not a significant correlation between the position of the first double bond and the $ΔV_{0.5}$ (Slope = 2.6 ± 1.4; $R^2 = 0.19$; p = 0.09) (Fig. 7H). There is no correlation between the position of the first double bond and the maximal conductance ($G_{max}$) (Supplemental Fig. 2D). However, there is a strong and significant correlation between the position of the
first double bond relative to the PUFA head and the apparent binding affinity for 
$I/I_0$ (double bonds closer to the head group give lower $K_m$) (Slope = $3.6 \pm 0.49$; $R^2$
$= 0.83$; $p < 0.0001$) (Fig. 7I). These data suggest that the location of the first
double bond does indeed contribute to the ability of PUFAs to increase $K^+$ current
with high apparent affinity for the $I_{Ks}$ channel.
Figure 2.8. Effects on and affinity for $I_{Ks}$ channel can be predicted using the position of the first double bond, demonstrated using 18:3Δ5,9,12. A-C) $K^+$ current in 0 μM (Left) and 70 μM (Right) for (A) 20:3Δ5,8,11, (B) 22:5Δ4,7,10,13,16, and (C) 18:3Δ5,9,12. D-F) Comparison of dose dependent (D) $I/I_0$ increase, (E) $I_{max}$, and (F) $V_{0.5}$ between 20:3Δ5,8,11, 22:5Δ4,7,10,13,16, and 18:3Δ5,9,12. G-I) Correlation between the position of the first double bond of all PUFAs and the (G) $I/I_0$ (Slope = -0.24 ± 0.1; $R^2$ = 0.27; p = 0.04), (H) $V_{0.5}$ (Slope = 2.6 ± 1.4; $R^2$ = 0.19; p = 0.09), and (I) $K_m$ of $I/I_0$ (Slope = 3.6 ± 0.49; $R^2$ = 0.83; p < 0.0001).

Figure 2.9. Correlations between PUFA parameters and $G_{max}$. A) Comparison of dose dependent increase in $G_{max}$ with all PUFAs. B) Correlation between the length of the PUFA tail and $G_{max}$ (Slope = 0.08 ± 0.04; $R^2$ = 0.25; p = 0.05). C) Correlation between the number of double bonds and $G_{max}$ (Slope = 0.12 ± 0.09; $R^2$ = 0.11; p = 0.21). D) Correlation between the position of the first double bond and $G_{max}$ (Slope = -0.05 ± 0.04; $R^2$ = 0.1; p = 0.22). E) Correlation between the position of the last double bond and $G_{max}$ (Slope = 0.05 ± 0.05; $R^2$ = 0.05; p = 0.42).
2.4. Hierarchical cluster analysis reveals distinct groupings of PUFAs according to their effects.

We grouped PUFAs with similar effects using hierarchical cluster analysis to determine key differences between the PUFAs that were tested (Wessa 2017). All four effects on the I$_{\text{Ks}}$ channel (i.e. $I/I_0$, $\Delta V_{0.5}$, $G_{\text{max}}$, and $K_m$ of $I/I_0$) were input simultaneously to be clustered according to similarity in all of the effects. We then looked at the PUFAs that were clustered together to determine whether there are similarities between those PUFAs in their structures (i.e. tail length, number of double bonds, position of the first double bond, and the position of the last double bond). Our hierarchical cluster analysis revealed three distinct groupings of PUFAs based on dissimilarity of PUFA effects between the groups (denoted by the height of dendrogram branches) (Fig. 8A). The first grouping (Cluster 1) included $18:3\Delta5,9,12$, $22:5\Delta4,7,10,13,16$, $20:3\Delta5,8,11$ and $20:3\Delta8,11,14$. All of these PUFAs have their double bonds grouped closer to the PUFA head and they produce the largest left-shifts in the $V_{0.5}$ of the I$_{\text{Ks}}$ channel. Interestingly, all of these PUFAs are $\omega$-6 and $\omega$-9 PUFAs. The next grouping (Cluster 2) includes $18:2\Delta9,12$, $22:4\Delta4,10,13,16$, $20:5\Delta5,8,11,14,17$, $22:6\Delta4,7,10,13,16,19$, $18:3\Delta6,9,12$. The following grouping (Cluster 3) includes $18:3\Delta9,12,15$, $22:5\Delta7,10,13,16,19$, $16:3\Delta7,10,13$, and $22:4\Delta7,10,13,16$. Clusters 2 and 3 both include PUFAs that have moderate effects on the I$_{\text{Ks}}$ channel. The final grouping (Cluster 4) includes $20:3\Delta11,14,17$, $22:3\Delta13,16,19$, and $14:3\Delta5,8,11$, all of which have the majority of their double bonds grouped closer to the end of the PUFA tail and produce little to no change in the $V_{0.5}$ of the I$_{\text{Ks}}$ channel. Interestingly, all
of these PUFAs are ω-3 PUFAs. Clusters 1 and 4 demonstrated the greatest dissimilarity in their effects. The hierarchical cluster analysis suggests that the PUFAs with double bonds located closer to the PUFA head (Cluster 1) resulted in the greatest left-shift in the $V_{0.5}$. In addition, the PUFAs with double bonds further from the PUFA head (Cluster 4) produced the smallest changes in the $V_{0.5}$, which is in line with our single regression analysis and conclusions. Most PUFAs in Clusters 2 and 3 were either ω-3 PUFAs with a double bond close to the head group or ω-6 PUFAs with no double bond close to the head group. These PUFAs had intermediate effects on the $I_{Ks}$ channel. The pattern that emerges from this analysis is that the most effective PUFAs on the $I_{Ks}$ channel are PUFAs with a double bond close to the head group (position 4 or 5), but that lack a double bond close to the end of the PUFA tail (ω-6 and ω-9 PUFAs). For example, when the first and last double bonds are both shifted closer to the end of the tail (i.e. shifted closer to the omega carbon) by comparing $22:5\Delta 4,7,10,13,16$ (Cluster 1) to $22:5\Delta 7,10,13,16,19$ (Cluster 3) the $I/I_0$ is reduced by nearly half. (Fig. 8B). Because the cluster analysis suggests that the ω-6 and ω-9 PUFAs produce the largest shifts in the $V_{0.5}$, we correlate the ω-number with each of the effects on the $I_{Ks}$ channel. There is a significant correlation between the ω-number and $I/I_0$ (Slope = 0.39 ± 0.13; $R^2 = 0.40$; $p = 0.009$) (Fig. 8C). In addition, there is a strong correlation between ω-number and the $\Delta V_{0.5}$, which is consistent with our findings from the dendrogram grouping (Slope = -6.0 ± 1.5; $R^2 = 0.54$; $p = 0.001$) (Fig. 8D). However, there is no correlation between the ω-number and the maximal conductance ($G_{max}$) (Sup.
Fig. 2E). There is also no correlation between the ω-number and apparent binding affinity (Slope = -1.0 ± 1.3; R² = 0.05; p = 0.45) (Fig. 8E).

Figure 2.10. Hierarchical clustering suggests three distinct groups of PUFAs and suggests role of ω-number in effects on Iₖₑₚ. A) Dendrogram of all PUFAs tested grouped according to their effects on the Iₖₑₚ channel. B) Comparison of dose dependent I/I₀ increase between 22:5Δ4,7,10,13,16 (Cluster 1) and 22:5Δ7,10,13,16,19 (Cluster 3). C-E) Correlation between the position of the last double bond of all PUFAs and (C) I/I₀ (Slope = 0.39 ± 0.13; R² = 0.40; p = 0.009), (D) ΔV₀.₅ (Slope = -6.0 ± 1.5; R² = 0.54; p = 0.001), and (E) Kₘ of I/I₀ (Slope = -1.0 ± 1.3; R² = 0.05; p = 0.45).
2.5. Multivariable regression analysis.

To test whether the PUFA effects – $I/I_0$, $\Delta V_{0.5}$, $G_{\text{max}}$, and $K_m$ of $I/I_0$ – depend on a combination of the different parameters (Length, number of double bonds, and location of the first and last double bonds) of the PUFA tails, we conducted a multivariable regression of each PUFA effect using combinations of the four PUFA parameters simultaneously (Sup. Fig. 3). The multivariable regression using all four parameters improved the fit for $I/I_0$ (Adjusted $R^2$ increased from 0.40 to 0.54) and $G_{\text{max}}$ (Adjusted $R^2$ increased from 0.25 to 0.60). According to the multivariable regression analysis $I/I_0$ is significantly correlated to the location of the first double bond (Table 2). $G_{\text{max}}$ is significantly correlated to the length of the tail, the number of double bonds and the location of the first and last double bond (Table 2). However, the $\Delta V_{0.5}$ and $K_m$ (for $I/I_0$) single regressions were not improved by the addition of more parameters of the PUFA. This suggests that the $\Delta V_{0.5}$ and $K_m$ (for $I/I_0$) are highly dependent on single PUFA parameters (i.e. the position of the last double bond and first double bond, respectively). To show the quality of the fit of the multivariable analysis, we show in Sup. Fig. 3 the measured and the predicted values for the fits of $I/I_0$ (Sup. Fig. 3A) and $G_{\text{max}}$ (Sup. Fig. 3B).
General Discussion

Neither the length of the carbon tail nor the number of double bonds in the tail showed a strong correlation with the effects on the $I_{Ks}$ currents or the affinity for the $I_{Ks}$ channel. However, there were correlations between having the first double bond closer to the PUFA head and increasing $I/I_0$ and improving the apparent binding affinity for $I/I_0$ (lower $K_m$). In contrast, having a double bond close to the end of the PUFA tail (i.e. an $\omega$-3 PUFA) decreased the effect of the PUFA on the $V_{0.5}$ of the $I_{Ks}$ channel, whereas having a double bond further away from the end of the PUFA tail (i.e. an $\omega$-6 or $\omega$-9 PUFA) increases the effect of the PUFA on the $V_{0.5}$. Using our data, the location of the first and the last double bonds,
relative to the PUFA head can therefore be used to develop effective PUFAs that bind with high affinity to the $I_{\text{Ks}}$ channel.

One of the main obstacles to the clinical use of PUFAs for cardiovascular diseases has been inconsistencies in clinical trials investigating the relationship between dietary fatty acids and cardiovascular disease risk. Two recent clinical studies report on correlation between ω-3 and ω-6 fatty acid supplements with cardiovascular disease risk (Aung et al. 2018; Virtanen et al. 2018). In a meta-analysis of 10 clinical trials looking at the relationship between ω-3 fatty acid supplements and the risk for cardiovascular disease and negative disease outcomes, Aung et al. reported that there is no significant correlation between the use of ω-3 fatty acid supplements and cardiovascular disease risk (Aung et al. 2018). In contrast, Virtanen et al. reported beneficial effects of ω-6 PUFAs on cardiovascular disease risk in the Kuopio Ischaemic Heart Disease Risk Factor Study (Virtanen et al. 2018). These findings are in agreement with our conclusions that ω-3 PUFAs have reduced left-shifting effects on the $I_{\text{Ks}}$ voltage-dependence whereas ω-6 PUFAs (along with ω-9 PUFAs) are the most beneficial for left-shifting the $I_{\text{Ks}}$ voltage dependence and increasing $I_{\text{Ks}}$ current.

Tian et al., 2016, applied PUFAs, such as DHA (22:6Δ4,7,10,13,16,19), onto Slo1-β1 BK channels to determine the important features for PUFAs to interact with BK channels (Tian et al. 2016). They concluded that double bonds at least halfway through the PUFA tail (at carbons 9-12) are important for a high affinity
interaction of PUFAs with Slo1 BK channels. They proposed that these double bonds allow more curvature of the PUFA molecule and make it more compact, thus increasing the stability of the PUFA in its binding site (Tian et al. 2016). In contrast, we find here that the double bonds closer to the PUFA head groups (at carbons 4-5) are important for the effect on and apparent binding affinity for the I_{Ks} channel. However, how the PUFA tail specifically interacts with the I_{Ks} channel to increase the apparent binding affinity is not known from this data and will be the focus of future studies.

Tian et al., 2016, proposed that the PUFA head group takes part in an anion-pi interaction with a tyrosine residue in the S6 segment of Slo1 BK channels (Tian et al. 2016). In contrast, we previously showed that the effects on the I_{Ks} channel are mainly due to an electrostatic interaction between the PUFA head group and the positively charged S4 segment of the I_{Ks} channel (Liin et al. 2015). PUFA effects on the BK channels are believed to be due to PUFAs in the inner leaflet of the membrane, whereas our effects on I_{Ks} channels are believed to be due to PUFAs in the outer leaflet of the membrane. This fact, along with their different putative site of DHA interaction, is indicative of different mechanisms for the PUFA effects on BK and I_{Ks} channels (intracellular action on S6 versus extracellular action on S4). Different mechanisms for PUFA effects on various ion channels is also supported by the effects of PUFAs on cardiac Na^+ and Ca^{2+} channels. For example, in contrast to the increase in currents shown here for DHA and EPA on I_{Ks} channels, DHA and EPA have been shown to reduce the
currents through voltage-gated Na\(^+\) and Ca\(^{2+}\) channels (Kang and Leaf 1996; Xiao et al. 1997; Xiao et al. 1995). This further suggests different mechanisms of action of PUFAs on different voltage-gated ion channels. Therefore, there is a possibility for channel-specific PUFA interactions which could be, in part, attributed to different features of the PUFA tail. Future studies would have to determine what PUFA features are important for other cardiac ion channels and whether channel-specific PUFAs could be developed.

Our current model for how PUFAs affect the I\(_{Ks}\) channel is that 1) the PUFA tail inserts into the lipid bilayer, 2) the PUFA then diffuses in the membrane to the I\(_{Ks}\) channel, 3) the PUFA tail binds to the I\(_{Ks}\) channel and anchors the PUFA head group near the positively charged voltage sensor of the I\(_{Ks}\) channel, 4) the negatively charged head group of the PUFA electrostatically attracts the voltage sensor and activates the I\(_{Ks}\) channel (Liin et al. 2015). The presently reported changes in the apparent PUFA affinity for the I\(_{Ks}\) channel due to variations in the PUFA tail are consistent with this model. However, in addition to changing the apparent PUFA affinity to the I\(_{Ks}\) channel, we here show that the positions of the double bonds of the PUFAs also affect the size of the left shift of the voltage dependence of the I\(_{Ks}\) channel (e.g. the position of the last double bond in the tail) and, in some instances, also affects the maximal conductance of the I\(_{Ks}\) channel. The reasons for these additional effects are not clear. It is possible that the interactions between the last double bonds and the I\(_{Ks}\) channel alter the location of the negatively charged head group of the PUFAs relative to the
charges in S4 and thereby cause bigger or smaller shifts in the voltage
dependence of activation.

The PUFAs were tested at pH 9, a pH at which the carboxyl head group of the
PUFA molecules interacting with the I_{KS} channel are expected to be deprotonated
and negatively charged, with no additional effects on normal wild type I_{KS} function
compared to pH 7.5 (Sup. Fig. 4). Our group has previously described that DHA
(22:6Δ4,7,10,13,16,19) increases Kv7.1 K⁺ current at pH 7.5, but that this effect
is abolished when Kv7.1 is co-expressed with the accessory β-subunit KCNE1
(Liin et al. 2015). The head groups of N-AT and DHA-glycine are more
efficacious than DHA on I_{KS} channels, because they remain more deprotonated
and negatively charged at physiological pH 7.5 Knowing that a PUFA head with a
lower pKa increases I_{KS} current at physiological pH = 7.5, we can now begin
making modifications in the tails of these PUFA analogues to improve the binding
affinity to the I_{KS} channel based on the data reported here. Understanding the
role of the double bonds for the PUFA tail in altering the binding affinity to the I_{KS}
channel will allow us to develop new PUFA analogues with better effect on I_{KS}
channels by attaching high affinity tails to head groups, such as taurine or
glycine, that are more deprotonated and efficacious at physiological pH.
The work presented here was performed using the *Xenopus laevis* oocytes expression system. This poses some limitations to understanding the effects of PUFAs on the physiological $I_{Ks}$ channel due to the absence of intrinsic factors in human cardiomyocytes that can influence the $I_{Ks}$ macromolecular complex. Ongoing and future experiments will determine the effects of the PUFAs examined here on the $I_{Ks}$ current present in human cardiomyocytes to further understand the therapeutic potential of PUFAs as anti-arrhythmic compounds. In addition, our experiments only shed light on the acute effects of PUFAs applied to the $I_{Ks}$ channel. Further experiments are needed to determine the long-term effects of chronic application of PUFAs to the $I_{Ks}$ channel.

**Figure 2.12.** pH does not influence the G-V of wild type $I_{Ks}$ channels. Comparison of the conductance vs. voltage relationship of $I_{Ks}$ channels in ND96 pH 7.5 and ND96 pH 9. ($V_{0.5}$ pH 7.4: 28.6 ± 1.8 mV, n=23; $V_{0.5}$ pH 9.0: 26.5 ± 1.8 mV, n=23.)
The next step in understanding the important properties of PUFAs for increasing $I_{Ks}$ current in LQTS is to test whether the PUFAs deemed effective here on wild type $I_{Ks}$ channels are able to reverse Long QT mutation phenotypes in $I_{Ks}$ channels. Mutations in the $I_{Ks}$ channel that cause LQTS type 1 (LQT1) cause loss-of-function in the $I_{Ks}$ channel by a variety of mechanisms, including a right-shift in the voltage dependence of the $I_{Ks}$ channel, changes in channel kinetics, or alterations in trafficking to the cell membrane (Bohnen et al. 2017; Huang et al. 2018; Liin et al. 2016). Here, we showed that ω-6 (and ω-9) PUFAs with double bonds close to the PUFA head produce the largest increases in $K^+$ current, shift the wild type G-V curve leftward the most, and activate the channel at more negative voltages. These data suggest that applying the same PUFAs to a mutated $I_{Ks}$ channel would restore the wild type $I_{Ks}$ current and, therefore, restore the normal ventricular action potential. A previous study from our group showed that a modified PUFA, N-arachidonoyl taurine (N-AT), could restore much of the defects of eight different LQTS-causing mutations, albeit at high N-AT concentrations (Liin et al. 2016). This presents a need for new PUFA analogues that can influence $I_{Ks}$ currents in a lower concentration range which would be more therapeutically relevant and reduce potential side effects on other channels. The findings reported here provide a foundation for the design of higher affinity PUFA analogues that can be used for the treatment of LQTS. Future experiments need to be done to test the best compounds found here on $I_{Ks}$ channels bearing LQT1-causing mutations. In addition, because different PUFAs shift the G-V curve by different amounts and different LQT1 mutations
Table 2.1: Summary of PUFA properties and effects on the le channel

<table>
<thead>
<tr>
<th>Name</th>
<th>Length</th>
<th>Number of Double Bonds</th>
<th>α / (μM)</th>
<th>ΔV α (mV)</th>
<th>G α max0 (pA)</th>
<th>ΔV β (mV)</th>
<th>G β max0 (pA)</th>
<th>ΔV 0.5 (mV)</th>
<th>G 0.5 max0 (pA)</th>
<th>ΔV 1 (mV)</th>
<th>G 1 max0 (pA)</th>
<th>ΔV 1.5 (mV)</th>
<th>G 1.5 max0 (pA)</th>
<th>ΔV 2 (mV)</th>
<th>G 2 max0 (pA)</th>
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<tbody>
<tr>
<td>Docosahexanoic acid</td>
<td>22</td>
<td>5</td>
<td>0.1</td>
<td>13.4</td>
<td>22.3 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>11.6 ± 0.2</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
<td>14 ± 6.0</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
<td>14 ± 6.0</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>Docosatetraenoic acid</td>
<td>22</td>
<td>5</td>
<td>0.1</td>
<td>5.9</td>
<td>10.0 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
<td>14 ± 6.0</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
<td>14 ± 6.0</td>
<td>0.2 ± 5.9</td>
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</tr>
<tr>
<td>Tetradecatrienoic acid</td>
<td>18</td>
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<td>4.9 ± 0.1</td>
<td>10.0 ± 0.1</td>
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<td>4.9 ± 0.1</td>
<td>14 ± 6.0</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
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<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
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<tr>
<td>Hexadecatrienoic acid</td>
<td>18</td>
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<td>4.9 ± 0.1</td>
<td>10.0 ± 0.1</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
<td>14 ± 6.0</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
<td>14 ± 6.0</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
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<tr>
<td>Saturated fatty acids</td>
<td>16</td>
<td>3</td>
<td>0.1</td>
<td>5.9</td>
<td>10.0 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>7.6 ± 0.1</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
<td>14 ± 6.0</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
<td>14 ± 6.0</td>
<td>0.2 ± 5.9</td>
<td>4.9 ± 0.1</td>
</tr>
</tbody>
</table>

All values for I/I, ΔV, G, and Kc for I/I, Vc, and Gc - represent the mean and standard error of the mean (SEM).

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**shift the G-V curves by different amounts in the opposite direction by matching each voltage axis, one could develop mutation-specific therapeutics by matching each PUFA with each mutation depending on their relative effects on the G-V curves.**
### Table 2.2: Multiple regression analysis results.

<table>
<thead>
<tr>
<th>Variables</th>
<th>$I/I_0$</th>
<th>$G_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_L$</td>
<td>$0.62 \pm 0.29$ ns</td>
<td>$0.39 \pm 0.09$ ***</td>
</tr>
<tr>
<td>$C_{#B}$</td>
<td>$-1.6 \pm 0.87$ ns</td>
<td>$-0.97 \pm 0.27$ **</td>
</tr>
<tr>
<td>$C_{FB}$</td>
<td>$-0.65 \pm 0.28$ *</td>
<td>$-0.34 \pm 0.09$ **</td>
</tr>
<tr>
<td>$C_{LB}$</td>
<td>$-0.26 \pm 0.33$ ns</td>
<td>$-0.33 \pm 0.10$ **</td>
</tr>
<tr>
<td>$C_0$</td>
<td>2.7</td>
<td>1.22</td>
</tr>
<tr>
<td>Adj. $R^2$</td>
<td>0.54</td>
<td>0.6</td>
</tr>
<tr>
<td>$F$</td>
<td>0.01</td>
<td>0.005</td>
</tr>
</tbody>
</table>

The measured outcomes ($I/I_0$ and $G_{\text{max}}$) were fitted to the equation $y = C_0 + C_L \times (\text{Tail length} - \text{Average Tail Length}) + C_{\#B} \times (\text{Number of double bonds} - \text{Average number of double bonds}) + C_{FB} \times (\text{Position of the first double bond} - \text{Average position of the first double bond}) + C_{LB} \times (\omega\text{-number} - \text{Average }\omega\text{-number})$. $C_0$ is the average response of all tested PUFAs. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ *** ns = not significant.
CHAPTER 3. Polyunsaturated fatty acids produce a range of activators for heterogeneous $I_{Ks}$ channel dysfunction

Background

The ventricular cardiac action potential is controlled by the activation of depolarizing and repolarizing ionic currents. One of the dominant repolarizing currents during the ventricular action potential is the slow delayed-rectifier potassium current ($I_{Ks}$), which is critical for the timing of action potential termination (Barhanin et al. 1996; Sanguinetti 1996; Salata et al. 1996). The $I_{Ks}$ channel underlies the slow component of the delayed rectifier $K^+$ current and is comprised of the voltage-gated $K^+$ channel, Kv7.1, $\alpha$-subunit and the KCNE1 accessory $\beta$-subunit (Barhanin et al. 1996; Salata et al. 1996; Sanguinetti 1996). Kv7.1 forms a tetrameric Kv channel that associates with the $\beta$-subunit, KCNE1, which dramatically alters the voltage dependence and kinetics of Kv7.1 channel activation and is necessary to generate the physiological $I_{Ks}$ current (Barhanin et al. 1996; Salata et al. 1996; Sanguinetti 1996; Barro-Soria et al. 2014; Osteen et al. 2010).

Ion channel mutations, or channelopathies, are the root of many pathological conditions, including the arrhythmogenic disorder Long QT Syndrome (LQTS) (Bohannon et al. 2017; Alders and Christiaans 2003; Schwartz, Crotti, and Insolia 2012). LQTS is an inherited disorder that is characterized by a prolonged QT

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interval – the time between ventricular depolarization and repolarization – on the electrocardiogram (ECG) (Schwartz, Crotti, and Insolia 2012; Waddell-Smith and Skinner 2016). The most common form of LQTS (LQT1) is caused by mutations in the voltage-gated K\(^+\) channel known as the I\(_{Ks}\) channel (Alders and Christiaans 2003; Roden 2008; Wu, Ding, and Horie 2016).

Treatment options for Long QT Syndrome include pharmacological attenuation of β-adrenergic stimulation by β blockers or the implantation of a cardioverter defibrillator (Cho 2016; Schwartz, Crotti, and Insolia 2012; Waddell-Smith and Skinner 2016). Though these treatments help to prevent arrhythmia or stop arrhythmia, they do not work for all individuals (Chockalingam et al. 2012; Schwartz, Crotti, and Insolia 2012) and they do not directly target the underlying channelopathies that lead to LQTS (Schwartz, Ackerman, and Wilde 2017). Therefore, there is a need for new therapeutics that directly target the channelopathies that lead to LQTS.

We have previously shown that the activity of the I\(_{Ks}\) channel can be modified by lipids, such as polyunsaturated fatty acids (PUFAs) (Liin et al. 2015; Liin et al. 2016). PUFAs and PUFA analogues are amphipathic molecules that have two distinct structural regions that can participate in interactions with membrane proteins: 1) a charged hydrophilic head group, and 2) a long, hydrophobic tail with two or more double bonds. PUFAs and PUFA analogues influence the activation of K\(^+\) channels through a lipoelectric mechanism in which the
hydrophobic tail integrates into the cell membrane near the voltage sensing domain and electrostatically attracts the positively charged S4 through its negatively charged hydrophilic head group, thus facilitating channel activation (Fig. 1A,B). We have recently demonstrated that in PUFAs with a carboxyl head group the position of the double bonds in the tail correlates significantly with apparent binding affinity to the Iₖₛ channel (Bohannon et al. 2018). Specifically, having the first double bond close to the carboxyl head group is important for high apparent binding affinity for the Iₖₛ channel and PUFA-induced enhancement of Iₖₛ current (Bohannon et al. 2018). It is known that a negatively charged head group is necessary for activation of voltage-gated K⁺ channels (Liin et al. 2015; Borjesson, Hammarstrom, and Elinder 2008). Docosahexaenoic acid (DHA), which can bear a negative charge at its carboxyl head group, shifts the voltage dependence of Kv7.1 channel activation to more negative voltages, however co-expression of KCNE1 abolishes DHA sensitivity (Liin et al. 2015). KCNE1 has recently been shown to tune PUFA sensitivity by inducing a conformational change of the S5-P-helix loop that results in protonation of the PUFA head group (Liin, Yazdi, et al. 2018). This protonation can be circumvented by using PUFA analogues that are negatively charged at physiological pH (pH 7.4), such as DHA-glycine or N-arachidonoyl taurine (N-AT) (Liin et al. 2015; Liin et al. 2016). In addition to an electrostatic effect on the voltage sensor of the Iₖₛ channel, our group has also recently demonstrated that PUFA analogues have an additional effect on the pore of the Iₖₛ channel (Liin, Yazdi, et al. 2018): a lysine residue (K326) in the S6 helix of the Iₖₛ channel
electrostatically interacts with the negatively charged head group of PUFA analogues and this electrostatic interaction increases the maximal conductance ($G_{\text{max}}$) of the cardiac $I_{\text{Ks}}$ channel (Liin, Yazdi, et al. 2018) (Fig. 1B-D).

A thorough characterization of PUFA analogues with a wide range of effects on the cardiac $I_{\text{Ks}}$ channel provides a means to develop novel treatments for LQT1-causing mutations of different severity. LQT1 is variable in its severity and can present with different symptoms based on the individual (Schwartz, Crotti, and Insolia 2012). For example, some patients carrying a mutation in KCNQ1 can have milder phenotypes associated with less severe prolongation of the QT interval. (Schwartz, Crotti, and Insolia 2012; Wu, Ding, and Horie 2016; Amin et al. 2011; Chouabe et al. 2000). For example, R533W, which causes a positive shift of approximately 15 mV in the voltage-dependence of activation, is associated with a milder cardiac phenotype (Chouabe et al. 2000). In other cases, such as for the KCNQ1 mutation A341V, that is one of the most severe presentations of LQT1, >30% of patients experience cardiac arrest or sudden cardiac death (Crotti et al. 2007; Schwartz, Crotti, and Insolia 2012). These examples highlight extreme differences in the manifestation of LQT1 in the clinical population that occur in a mutation-specific manner. Treatment for such distinct phenotypes would require an individualized approach. For this reason, there is a need to find new ways in which the effects of PUFA analogues can be tuned, allowing for more personalized treatment options for patients with LQT1. The purpose of the present study is to evaluate different PUFA head groups to
determine if the activating effects of PUFA analogues can be enhanced or attenuated through modifications to the charged PUFA head group.

Results

3.1. Linoleoyl-taurine and linoleoyl-glycine increase the \( I_{Ks} \) current by differentially affecting the \( V_{0.5} \) and the \( G_{max} \).

We have previously demonstrated that the negative charge of a PUFA with a carboxyl head group is neutralized by the presence of KCNE1 in \( I_{Ks} \) channels (Larsson, Larsson, and Liin 2018). For this reason, PUFAs with a carboxyl head group tend to have little effect on \( I_{Ks} \) channel activation at physiological pH. In this study, we investigate the effects of other head groups that are expected to promote \( I_{Ks} \) channel activation through the lipoelectric mechanism (Fig. 1A-B) with effects on the voltage sensor (Fig. 1A) and the pore (Fig. 1B). We compare the effects between PUFAs with varying functional groups of the hydrophilic PUFA head, but with the same hydrocarbon tail. To do this, we use two-electrode voltage clamp and a series of depolarizing voltage steps to measure the effects of PUFAs on \( I_{Ks} \) current (Fig. 1C). This allows us to measure the effects on the normalized current at 0 mV (\( I/I_0 \)), the shift in voltage dependence of \( I_{Ks} \) channel activation (\( \Delta V_{0.5} \)), and the maximal conductance (\( G_{max} \)) (Fig. 1D).
Figure 3.1. Illustration of the lipoelectric mechanism and measured effects on the cardiac $I_{Ks}$ channel. A) Schematic side view of the $I_{Ks}$ channel with S4 in green. Illustration of the electrostatic interaction of PUFA analogue (yellow) with the voltage sensor (green) of the cardiac $I_{Ks}$ channel, which leads to potentiation of upward S4 movement. B) Schematic top view of the $I_{Ks}$ channel with Kv7.1 in blue KCNE1 in purple. Illustration of the electrostatic interaction of PUFA analogue (yellow) with the positively charged lysine residue K326 in the S6 segment of the cardiac $I_{Ks}$ channel, which leads to an increase in the maximal conductance ($G_{max}$) of the $I_{Ks}$ channel. C) Activation protocol for the cardiac $I_{Ks}$ channel using two-electrode voltage clamp and raw current traces in 0 μM PUFA analogue (left) and 20 μM PUFA analogue (right) with arrows indicating tail currents. Red trace occurs at 20 mV for visualization of PUFA-induced increases in current. D) Representative current vs voltage relationship in 0 μM (black line) and 20 μM PUFA (blue line) highlighting increase in $I/I_0$ at 0 mV, leftward shift in the $V_{0.5}$, and increase in $G_{max}$ denoted by arrows.
We first compare three PUFAs and PUFA analogues that have a linoleic acid tail: linoleic acid, linoleoyl glycine (lin-glycine), and linoleoyl taurine (lin-taurine) (Fig. 2A-C). Application of 20 μM linoleic acid (Fig. 2A), which has a carboxyl head group, does not increase in I/I₀ (0.5 ± 0.1) (Fig. 2D), does not left-shift the V₀.₅ of Iₖ₉ channel activation (4.7 ± 0.9 mV) (Fig. 2F), and does not increase the Gₘₐₓ (0.7 ± 0.1) (Fig. 2H). Lin-glycine, when applied at 20 μM (Fig. 2B), produces a moderate increase in I/I₀ (5.3 ± 0.5) (Fig. 2D) and a moderate shift in the V₀.₅ (-26.4 ± 4.4 mV) (Fig. 2F), and produces the largest increase in the Gₘₐₓ (2.4 ± 0.2) (Fig. 2H). Lin-taurine, when applied at 20 μM, (Fig. 2C) produces the largest increase in I/I₀ (10.4 ± 4.0) (Fig. 2D) and largest left-shift in the V₀.₅ (-73.1 ± 2.6 mV) (Fig. 2F), and increases the Gₘₐₓ (2.0 ± 0.6) (Fig. 2H). Statistical analysis of the fitted parameters of the dose response curves show that lin-taurine has the biggest increase in I/I₀ (Fig. 2E) and V₀.₅ (Fig. 2G), whereas lin-glycine has the biggest increase in Gₘₐₓ (Fig. 2I). The size of the voltage shifts caused by the three PUFAs correlates with the predicted protonation (i.e. charge) of the different head groups (from the pKa values estimated for carboxyl, glycine, and taurine head groups in the lipid bilayer) at physiological pH (Table 1). In contrast, the effects on Gₘₐₓ did not correlate with the predicted charge of the PUFA head groups.
<table>
<thead>
<tr>
<th>PUFA</th>
<th>pKa&lt;sub&gt;1&lt;/sub&gt;</th>
<th>pKa&lt;sub&gt;2&lt;/sub&gt;</th>
<th>pKa&lt;sub&gt;3&lt;/sub&gt;</th>
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<tr>
<td>Linoleic acid</td>
<td>8.5</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Lin-glycine</td>
<td>7.6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Lin-taurine</td>
<td>2.7</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Lin-glycine+1C</td>
<td>8.0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Lin-glycine+2C</td>
<td>8.0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Lin-aspartate</td>
<td>7.6</td>
<td>9.1</td>
<td>NA</td>
</tr>
<tr>
<td>Lin-cysteic acid</td>
<td>2.6</td>
<td>7.1</td>
<td>NA</td>
</tr>
<tr>
<td>Lin-AP3</td>
<td>5.0</td>
<td>7.7</td>
<td>11.8</td>
</tr>
<tr>
<td>DHA</td>
<td>8.3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>DHA-glycine</td>
<td>7.5</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>DHA-taurine</td>
<td>2.8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
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<tr>
<td>Pinoleoyl taurine</td>
<td>2.8</td>
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</table>

Estimated pKa values for PUFAs and PUFA analogues associated with the cardiac I<sub>Ks</sub> channel were calculated by adding a factor of 3.5 to the starting pKa value calculated in solution.
Figure 3.2. Linoleoyl taurine produces the most potent activation of the $I_{Ks}$ channel compared to linoleoyl glycine and linoleic acid. A-C) Structure of and raw current traces measured in 0 μM (left) and 20 μM (right) A) linoleic acid, B) linoleoyl glycine, and C) linoleoyl taurine. D) Dose dependent effects of linoleic acid (n = 5), lin-glycine (n = 4), and lin-taurine (n = 3) on $I_{Ks}$ current ($I/I_0$) (mean ± SEM at maximal concentration). E) Statistical differences on $I/I_0$ effects ($I/I_0$ fitted from the dose response curve) measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis. F) Dose dependent effects of linoleic acid, lin-glycine, and lin-taurine on $I_{Ks}$ voltage dependence ($\Delta V_{0.5}$). G) Statistical differences on $\Delta V_{0.5}$ effects ($\Delta V_{0.5}$ fitted from the dose response curve) measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis. H) Dose dependent effects of linoleic acid, lin-glycine, and lin-taurine on $I_{Ks}$ maximal conductance ($G_{max}$). I) Statistical differences on $G_{max}$ effects ($G_{max}$ fitted from the dose response curve) measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis.
3.2. The different effects of lin-glycine and lin-taurine on the $V_{0.5}$ are not due to differences in the lengths of the PUFA head groups.

One structural difference between the head groups of lin-glycine and lin-taurine is that the glycine group is shorter in length compared to the taurine group (Fig. 2B-C). Therefore, we explored whether the different lengths of the head groups could explain the different activating effects of the two PUFAs on the $I_{Ks}$ channel. To do so, we inserted additional carbons into the head group of lin-glycine to elongate the glycine head group and then compared the effects of lin-glycine, lin-glycine+1C (Fig. 3A), and lin-glycine+2C (Fig. 3B). With the insertion of one additional carbon in the glycine head group, lin-glycine+1C has a similar length as lin-taurine. Application of lin-glycine produces an $I/I_0$ increase of $5.3 \pm 0.5$, whereas lin-glycine+1C and+2C surprisingly produce a smaller increase in $I/I_0$ ($1.7 \pm 0.1$ and $1.6 \pm 0.1$, respectively) (Fig. 3C and I). In addition, lin-glycine produced the largest shift in $V_{0.5}$ (-26.4 ± 4.4 mV) compared to lin-glycine+1C (-7.2 ± 2.5 mV) and lin-glycine+2C (-8.7 ± 0.5 mV) (Fig. 3D and J). Lin-glycine increases the $G_{\text{max}}$ of the $I_{Ks}$ channel (2.4 ± 0.2), whereas lin-glycine+1C and lin-glycine+2C produce no change in the $G_{\text{max}}$ (1 ± 0.1 and 0.9 ± 0.1, respectively) (Fig. 3E and K).

One possible mechanism behind the decreased effects of lin-glycine+1C and lin-glycine+2C, compared to lin-glycine, is that the addition of carbons in the glycine head group shifts the pKa of the head group, which thereby promotes protonation and loss of the negative charge in the head group. We therefore repeated the
experiments with lin-glycine, lin-glycine+1C, and lin-glycine+2C at pH 9. We have previously demonstrated, using PUFAs with a carboxyl head group, that conducting experiments at pH 9 can deprotonate the head group to restore the negative charge of the head group and allow PUFAs to activate the I\(K_s\) channel (Bohannon et al. 2018; Liin et al. 2015). Changing the solution from pH 7.5 to pH 9 does not alter the normal activation of the I\(K_s\) channel (Supp. Fig. S1). At pH 9, lin-glycine, lin-glycine+1C, and lin-glycine+2C all produce a similar left-shift in the voltage-dependence of I\(K_s\) channel activation at 20 μM (-43.6 ± 1.6 mV, -41.7 ± 1.7 mV, and -47.9 ± 2.4 mV, respectively) (Fig. 3G and J). Note that, at pH 9, application of lin-glycine results in a larger left-shift in the voltage dependence of the I\(K_s\) channel compared to the left-shifting effect of lin-glycine at pH 7.5 (Fig. 3J). This is consistent with our estimated pKa = 7.6 for lin-glycine: at pH 7.5, 50% of lin-glycine will be negatively charged, whereas, at pH 9, lin-glycine is almost fully in its deprotonated and negatively charged form. Lin-glycine displays higher apparent affinity and begins to shift the \(V_{0.5}\) at lower concentrations compared to lin-glycine+1C and lin-glycine+2C (Fig. 3G). Although the left-shifting effects of lin-glycine, lin-glycine+1C, and lin-glycine+2C were improved at pH 9 (Fig. 3G and J), all three PUFA analogues decrease the maximal conductance (\(G_{\text{max}}\)) of the channel (0.6 ± 0.1, 0.9 ± 0.1, and 0.5 ± 0.1, respectively) at pH 9 (Fig. 3H and K). The reason for this decrease in \(G_{\text{max}}\) is unclear. At pH 9, lin-glycine, lin-glycine+1C, and lin-glycine+2C all increase \(I/I_0\) (2.3 ± 0.1, 2.7 ± 0.3, and 1.8 ± 0.04, respectively) (Fig. 3F and I).
Figure 3.3. Increasing the length of the linoleoyl glycine head group alters the pKₐ and reduces activating effect on the Iₖₛ channel. A-B) Structure of A) linoleoyl glycine with the addition of one carbon in the head group (lin-glycine+1C) and B) linoleoyl glycine with the addition of two carbons in the head group (lin-glycine+2C). C-H) Dose dependent effects of lin-glycine (black dashed line) (n = 4), lin-glycine+1C (n = 3), and lin-glycine+2C (n = 3) on C) Iₖₛ current (I/I₀) at pH 7.5, D) Iₖₛ voltage dependence (ΔV₀.5) at pH 7.5, E) Iₖₛ maximal conductance (Gₘₐₓ) at pH 7.5, F) I/I₀ at pH 9, G) ΔV₀.5 at pH 9, and H) Gₘₐₓ at pH 9 (mean ± SEM at maximal concentration). I-K) Significant differences at 20 μM on I) I/I₀ effect, J) ΔV₀.5 effect, and K) Gₘₐₓ effect measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis.
The fact that the voltage-shifting effect of lin-glycine+1C and lin-glycine+2C are similar to that of lin-glycine at pH 9, but smaller at pH 7.5, suggest that the addition of 1 and 2 additional carbons in the glycine head group shifts the pKa of the glycine head group and reduces the likelihood that the glycine head will be deprotonated and negatively charged at pH 7.5. The size of the voltage shifts for lin-glycine+1C and lin-glycine+2C at pH 7.5 (50% smaller than for lin-glycine) are consistent with our estimated pKa values of lin-glycine+1C and lin-glycine+2C, which are both approximately 8.0 compared to 7.6 for lin-glycine (Suppl. Table 1). That the voltage-shifting effect of lin-glycine, lin-glycine+1C, and lin-glycine+2C at pH 9 are all similar suggests that it is not the length of the head group that renders lin-taurine more effective than lin-glycine, but mainly the protonation state of the PUFA head groups.

Figure 3.4. Current vs. voltage relationship between pH 7.5 and pH 9. Current-voltage relationship of I_Ks channel in pH 7.5 (black squares; mean ± SEM; n = 4) and pH 9.0 (red circles; mean ± SEM; n = 4).
3.3. Increasing the number of potentially charged moieties on the PUFA head group did not further promote $I_{Ks}$ channel activation.

Because we previously found that the charge of the head group is important for activating the cardiac $I_{Ks}$ channel, we tested whether it is possible to further improve the activating effects of PUFA analogues by increasing the charge available on the PUFA head group. To do so, we compared PUFA analogues that have 1 possible charge (lin-taurine and lin-glycine), 2 possible charges (lin-aspartate and lin-cysteic acid) (Fig. 4A, B), and 3 possible charges (lin-AP3) (Fig. 4C). Interestingly, increasing the number of potentially negatively charged groups on the PUFA head group did not further improve the effects on $I/I_0$, $V_{0.5}$, or $G_{max}$. Lin-aspartate, which has 2 potentially charged moieties, moderately increases $I/I_0$ ($4.0 \pm 0.1$) (Fig. 4D), moderately left-shifts the $V_{0.5}$ ($-34.5 \pm 2.3$ mV) (Fig. 4E), and moderately increases the $G_{max}$ ($1.4 \pm 0.1$) (Fig. 4F). The effects of lin-aspartate were similar to the effects of lin-glycine, which has only one potentially charged moiety (Fig. 4G-I). Lin-cysteic acid (Fig. 4C), which also possesses two potentially charged moieties, increases substantially $I/I_0$ ($9.2 \pm 0.4$) (Fig. 4D), substantially left-shifts the $V_{0.5}$ of $I_{Ks}$ channel activation ($-58.4 \pm 2.8$ mV) (Fig. 4E), and substantially increases the $G_{max}$ ($2.0 \pm 0.2$) (Fig. 4F). The effects of lin-cysteic acid were similar to the effects of lin-taurine, which has only one potentially charged moiety (Fig. 4G-I). Lastly, lin-AP3, which has 3 potential negative charges, produces the smallest increase in $I/I_0$ ($1.8 \pm 0.3$) (Fig. 4D and G), the smallest left-shift in the $V_{0.5}$ ($-5.7 \pm 1.3$ mV) (Fig. 4E and H), and produces no change in the $G_{max}$ ($1.1 \pm 0.1$) (Fig. 4F and I). Together these data show that
having >1 potentially charged moiety of the head group does not necessarily improve the efficacy of PUFA analogues, leading us to concentrate on glycine and taurine head groups as potential therapeutics for LQTS.
3.4. Taurine compounds have the largest current increase and left-shifting effect on the \(I_{Ks}\) channel.

We next compare PUFAs and PUFA analogues that have a docosahexaenoic acid (DHA) or pinolenic acid tail group to determine if the efficacy of glycine and taurine head groups are consistent across PUFA tail groups. DHA, which has a carboxyl head group, produces little change in \(I_{Ks}\) current at 20 \(\mu\)M (Fig. 5A) and produces a slight increase in \(I/I_0\) (2.0 ± 0.6) (Fig. 5D). DHA-glycine, which has a glycine head group produces a larger increase in \(I/I_0\) (4.7 ± 1.3 at 20 \(\mu\)M) relative to DHA. DHA-taurine produces the most robust increases in \(I_{Ks}\) current at 7 \(\mu\)M compared to PUFA analogues with a DHA tail, increasing \(I/I_0\) by 5.1 ± 0.7 at 7 \(\mu\)M (Fig. 5D). Surprisingly, at concentrations higher that 7 \(\mu\)M (20 \(\mu\)M), DHA-taurine decreases the current for reasons that are unclear. For this reason, we report the effects observed at 7 \(\mu\)M. When measuring the effects on the \(V_{0.5}\) of the \(I_{Ks}\) channel, DHA does not left-shift the \(V_{0.5}\) (0.1 ± 1.4 mV) (Fig. 5F), DHA-glycine has a moderate left-shifting effect (-16.5 ± 1.3 mV at 20 \(\mu\)M), and DHA-taurine has a more robust left-shifting effect (-45.3 mV ± 2.9 mV at 7 \(\mu\)M) (Fig. 5F). DHA,
DHA-glycine, and DHA-taurine all increase the $G_{\text{max}}$ (1.7 ± 0.3 at 20 μM, 2.0 ± 0.2 at 20 μM and 1.7 ± 0.1 at 7 μM, respectively) (Fig. 5H). Statistical analysis of the fitted parameters of the dose response curves show that DHA-taurine has the biggest increase in $V_{0.5}$ (Fig. 5G), whereas DHA-glycine has the biggest increase in $I/I_0$ (Fig. 5E) and $G_{\text{max}}$ (Fig. 5I).
Figure 3.6. DHA-taurine at 7 μM produces the most potent activation of the \( I_{Ks} \) channel compared to DHA-glycine and DHA at 20 μM. A-C) Structure of and raw current traces measured in 0 μM (left) and 20 μM (right) A) docosahexaenoic acid (DHA), B) docosahexanoyl glycine (DHA-glycine), and C) docosahexanoyl taurine (DHA-taurine, 0 μM (left) and 7 μM (right)). We report effects of DHA-taurine at 7 μM due to an unclear reduction in current caused by the application of 20 μM. D) Dose dependent effects of DHA (n = 4), DHA-glycine (n = 4), and DHA-taurine (n = 3) on \( I_{Ks} \) current (\( I/I_0 \)) (mean ± SEM at maximal concentration). E) Statistical differences on \( I/I_0 \) effects (\( I/I_0 \) fitted from the dose response curve) measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis. F) Dose dependent effects of DHA, DHA-glycine, and DHA-taurine on \( I_{Ks} \) voltage dependence (\( \Delta V_{0.5} \)). G) Statistical differences on \( \Delta V_{0.5} \) effects (\( \Delta V_{0.5} \) fitted from the dose response curve) measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis. H) Dose dependent effects of DHA, DHA-glycine, and DHA-taurine on \( I_{Ks} \) maximal conductance (\( G_{\text{max}} \)). I) Statistical differences on \( G_{\text{max}} \) effects (\( G_{\text{max}} \) fitted from the dose response curve) measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis.

Application of 20 μM pinolenic acid (Fig. 6A), which has a carboxyl head group, increases \( I/I_0 \) slightly (1.5 ± 0.3) (Fig. 6D), has little left-shifting effect on the \( V_{0.5} \) of \( I_{Ks} \) channel activation (-6 ± 1.8 mV) (Fig. 6F), and produces a slight increase in the \( G_{\text{max}} \) (1.4 ± 0.2) (Fig. 6H). Application of 20 μM pin-glycine (Fig. 6B), produces a moderate increase in \( I/I_0 \) (Fig. 6D) (3.8 ± 0.2), has a moderate left-shifting effect on the \( V_{0.5} \) of \( I_{Ks} \) channel activation (-21.1 ± 2.5) (Fig. 6F), and increases the \( G_{\text{max}} \) (1.8 ± 0.1) (Fig. 6H). Application of 20 μM pin-taurine (Fig. 6C) produces a robust increase in \( I/I_0 \) (Fig. 6D) (9.0 ± 1.4), potently left-shifts the \( V_{0.5} \) of \( I_{Ks} \) channel activation (-51.6 ± 3.5 mV) (Fig. 6F), and increases the \( G_{\text{max}} \) (1.9 ± 0.3) (Fig. 6H) relative to other PUFA analogues with a pinolenic acid tail. Statistical analysis of the fitted parameters of the dose response curves show that pin-taurine has the biggest increase in \( I/I_0 \) (Fig. 6E) and \( V_{0.5} \) (Fig. 6G),

Note: The text above is a natural representation of the document content as per the given guidelines.
whereas there were no significant differences in $G_{\text{max}}$ among the three compounds (Fig. 6I).
As previously mentioned, DHA-taurine produces an unexpected decrease in $I/I_0$ and $G_{\text{max}}$ at 20 μM. The largest effect on the $G_{\text{max}}$ induced by DHA-taurine on the $I_{ks}$ channel occurs at 7 μM, followed by a drastic decrease in $G_{\text{max}}$ at 20 μM. We have also observed a similar decrease in the $G_{\text{max}}$ at 20 μM with pin-taurine, however, this decrease in $G_{\text{max}}$ is not as pronounced as we see with 20 μM DHA taurine. The source of the reduction in $G_{\text{max}}$ with the application of some taurine compounds is not known. One possibility is that it is caused by a steric effect of the longer taurine head group resulting in obstruction of the $I_{ks}$ channel pore. To determine whether the reduction in $G_{\text{max}}$ is intrinsic to the taurine head group, we applied 100 μM taurine to the $I_{ks}$ channel. However, 100 μM taurine alone does not change $I/I_0$, $\Delta V_{0.5}$, or the $G_{\text{max}}$ (Suppl. Fig. S2) suggesting that the taurine head group alone is not responsible for the reduction in $G_{\text{max}}$. Therefore, PUFA-induced decreases in $G_{\text{max}}$ at concentrations ≥ 20 μM must be due to a different mechanism that occur through the combination of the taurine head group and the PUFA tail.
We directly compare the effects of PUFA analogue head groups across different PUFA tails to see if there were any differences in apparent binding affinity or effects on $I/I_0$, $\Delta V_{0.5}$, or $G_{\text{max}}$ depending on the tail. Our previous data and the data in this study suggest that PUFA analogues with glycine head groups have a pKa of ~7.5-7.6 when associated with $I_{ks}$ channels, suggesting that half the PUFA molecules with a glycine head group will be deprotonated and negatively

**Figure 3.8. Reduction of $I_{ks}$ current by the application of DHA taurine is not intrinsically related to the taurine head group alone.**

A) Structure of taurine and raw current traces measured in 0 μM taurine (left) and 100 μM taurine (right). B) Current vs voltage relationship in control (0 μM taurine) and following the addition of 100 μM taurine (mean ± SEM; $n = 4$).
charged at pH 7.5. PUFA analogues with a glycine head group produce similar max effects on $I/I_0$ (Fig. 7A) and $\Delta V_{0.5}$ (Fig. 7B), whereas $G_{\text{max}}$ was more varied (Fig. 7C). PUFA analogues with taurine head groups have an estimated pKa of $\sim 2.6$, suggesting that all of the PUFA molecules with a taurine head group will be deprotonated and negatively charged at pH 7.5. PUFA analogues with a taurine head group all produced much larger effects on $\Delta V_{0.5}$ than those with glycine head groups (Fig. 7B), whereas the effects on $G_{\text{max}}$ were all in a relative similar range (Fig. 7C). Lin-taurine and pinoleoyl-taurine produced much larger effects on $I/I_0$ than those with glycine head groups, whereas DHA-taurine produced a similar effect on $I/I_0$ as those with glycine head groups (Fig. 7A). In summary, the major difference between PUFAs with taurine and glycine head groups is in the effects on the $\Delta V_{0.5}$. This difference is mainly due to the pKa (i.e. the charge) of the PUFA head group, with little influence from the hydrophobic PUFA tail groups.

Figure 3.9. Comparison of effects by glycine head groups and taurine head groups on $I_{K_{\text{s}}}$ current ($I/I_0$), voltage dependence ($\Delta V_{0.5}$), and maximal conductance ($G_{\text{max}}$). A-C) Dose dependent effects of DHA-glycine ($n = 4$), lin-glycine ($n = 4$), pin-glycine ($n = 3$), DHA-taurine ($n = 3$), lin-taurine ($n = 3$), and pin-taurine ($n = 4$) on A) $I_{K_{\text{s}}}$ current ($I/I_0$), B) $I_{K_{\text{s}}}$ voltage dependence ($\Delta V_{0.5}$), and C) $I_{K_{\text{s}}}$ maximal conductance ($G_{\text{max}}$) (mean ± SEM at maximal concentration).
3.5. Hierarchical cluster analysis groups PUFA analogues that have similar functional effects.

We used hierarchical cluster analysis as an unbiased method to group PUFAs and PUFA analogues according to similarity of their effects on $I/I_0$, $\Delta V_{0.5}$, and $G_{\text{max}}$ at 20 µM (Fig. 8 and Suppl. Table 2). The hierarchical cluster analysis resulted in three distinct clusters of PUFAs and PUFA analogues. The first branch point results in the most distinct cluster (Cluster 1) of PUFA analogues that include lin-taurine, lin-cysteic acid, pin-taurine, and DHA-taurine which have the largest effects on the $V_{0.5}$. The second branch point divides clusters 2 and 3. Cluster 2 includes lin-aspartate, pin-glycine, DHA-glycine, and lin-glycine, which have intermediate effects on $I/I_0$ and the $G_{\text{max}}$. Cluster 3 includes linoleic acid, DHA, lin-AP3, and pinolenic acid, which have the smallest effects on $I_{K_s}$ channel activation. The results of the hierarchical cluster analysis suggest that PUFA analogues with a glycine head group have the most consistent effects on increasing $G_{\text{max}}$ and PUFA analogues with a taurine head group are most consistent in left-shifting the voltage dependence of $I_{K_s}$ channel activation.
Figure 3.10. Hierarchical cluster analysis and heat map demonstrate that taurine head groups are most similar in their voltage-shifting effects and glycine head groups are most similar in their effects on $G_{\text{max}}$. The dendrogram displays groupings of PUFAs and PUFA analogues according to similarity of their effects. The heat map displays the magnitude of the effects, with warmer colors representing PUFAs and PUFA analogues that have larger relative effects (closer to 1.0) on $I/I_0$, $G_{\text{max}}$, and $\Delta V_{0.5}$ and cooler colors representing PUFAs and PUFA analogues with smaller relative effects (closer to 0.0).
Table 3.2: Summary of effects of PUFA analogues on the cardiac $\mathcal{I}_{\text{Ks}}$ channel

<table>
<thead>
<tr>
<th>PUFA Name</th>
<th>$I/I_0$</th>
<th>$\Delta V_{0.5}$ (mV)</th>
<th>$G_{\text{MAX}}/G_{\text{MAX0}}$</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic acid</td>
<td>0.5 ± 0.04</td>
<td>4.7 ± 0.9</td>
<td>0.7 ± 0.04</td>
<td>5</td>
</tr>
<tr>
<td>Lin-glycine</td>
<td>5.3 ± 0.5</td>
<td>-26.4 ± 4.4</td>
<td>2.4 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>Lin-taurine</td>
<td>10.4 ± 4.0</td>
<td>-73.1 ± 2.6</td>
<td>2.0 ± 0.6</td>
<td>3</td>
</tr>
<tr>
<td>Lin-glycine+1C</td>
<td>1.7 ± 0.2</td>
<td>-7.2 ± 2.5</td>
<td>1.3 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td>Lin-glycine+2C</td>
<td>1.9 ± 0.4</td>
<td>-8.7 ± 0.5</td>
<td>1.1 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td>Lin-cysteic acid</td>
<td>9.2 ± 0.4</td>
<td>-58.4 ± 2.8</td>
<td>2.0 ± 0.2</td>
<td>5</td>
</tr>
<tr>
<td>Lin-aspartate</td>
<td>4.0 ± 0.1</td>
<td>-34.5 ± 2.3</td>
<td>1.4 ± 0.1</td>
<td>4</td>
</tr>
<tr>
<td>Lin-AP3</td>
<td>1.8 ± 0.3</td>
<td>-5.7 ± 1.3</td>
<td>1.1 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>DHA</td>
<td>2.0 ± 0.6</td>
<td>0.1 ± 1.4</td>
<td>1.7 ± 0.3</td>
<td>4</td>
</tr>
<tr>
<td>DHA-glycine</td>
<td>4.7 ± 1.3</td>
<td>-16.5 ± 1.3</td>
<td>2.0 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>DHA-taurine</td>
<td>5.1 ± 0.7</td>
<td>-45.3 ± 2.9</td>
<td>1.7 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>Pinolenic acid</td>
<td>1.5 ± 0.3</td>
<td>-6 ± 1.8</td>
<td>1.4 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td>Pin-glycine</td>
<td>3.8 ± 0.2</td>
<td>-21.1 ± 2.5</td>
<td>1.8 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>Pin-taurine</td>
<td>9.0 ± 1.4</td>
<td>51.6 ± 3.5</td>
<td>1.9 ± 0.3</td>
<td>4</td>
</tr>
</tbody>
</table>

Summary of the effects of PUFAs on $I_{\text{Ks}}$ $I/I_0$, $\Delta V_{0.5}$ (mV), and $G_{\text{MAX}}/G_{\text{MAX0}}$ with the number of experiments (N). Data is represented as mean ± SEM at the maximum concentration used (effects of DHA-taurine are reported at 7 µM due to a decrease in current observed at 20 µM).
3.6. Circulating concentrations of Lin-glycine with albumin and other fatty acids promotes the activation of the cardiac $I_{Ks}$ channel by left-shifting the voltage dependence of activation.

In the body, PUFAs circulate in complex with serum albumin but interact with channel proteins in the free fatty acid form. In addition, PUFAs in the bloodstream will be in circulation with other types of fatty acids, including monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs). In order to emulate the effects of PUFAs under physiological conditions, we applied lin-glycine in combination with the MUFA oleic acid, the SFA stearic acid, and albumin (Tsukamoto and Sugawara 2018; Abdelmagid et al. 2015). We applied 0.2 mM albumin/ 0.7 mM lin-glycine/ 0.6 mM oleic acid/ 0.6 mM stearic acid which we refer to as albumin + fatty acids (Abdelmagid et al. 2015; Tsukamoto and Sugawara 2018). Following the application of albumin + fatty acids, we see an increase in $I_{Ks}$ current (Fig. 9A). In the current vs voltage relationship, we observe that the application of albumin alone causes a slight, but not significant, decrease in the current (Fig. 9B). However, with the application of albumin + fatty acids, we observe an increase in the current (such that the current is restored to the current seen under control conditions), as well as a leftward shift in the voltage dependence of $I_{Ks}$ activation (Fig. 9B). Lin-glycine in combination with MUFAs, SFAs, and albumin produces a significant increase in $I_{Ks}$ current (3.4 ± 0.6) compared to control ($p = 0.007$) and albumin alone ($p = 0.004$) (Fig. 9C). In addition, we observe a leftward shift in the voltage-dependence of $I_{Ks}$ activation (-20.1 ± 5.1 mV) compared to control ($p = 0.007$) and the application of albumin
alone ($p = 0.006$), however it does not significantly increase the maximal conductance of the $I_{Ks}$ channel ($0.94 \pm 0.01$) ($p = 0.5$) (Fig. 9D-E). These data together suggest that there is still a substantial concentration of lin-glycine in the free fatty acid form that is available to promote the activation of the cardiac $I_{Ks}$ channel by left-shifting the voltage dependence of activation.

Figure 3.11. Lin-glycine, in combination with physiological concentrations of monounsaturated and saturated fatty acids and albumin, promotes the activation of the $I_{Ks}$ channel. A) Raw current traces measured in control ND96 (left) and in the presence of $0.2$ mM albumin + $0.7$ mM Lin-glycine/$0.6$ mM Oleic acid/$0.6$ mM Stearic acid (Fatty Acids) (right). B) Current-voltage relationship of cells in control ND96 (black squares), $0.2$ mM albumin alone (red circles) and $0.2$ mM albumin + $0.7$ mM Lin-glycine/$0.6$ mM Oleic acid/$0.6$ mM Stearic acid (green triangles) (mean ± SEM; $n = 3$). C) Statistical differences on $I/I_0$ effects ($I/I_0$ fitted from the dose response curve) measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis. D) Statistical differences on $\Delta V_{0.5}$ effects ($\Delta V_{0.5}$ fitted from the dose response curve) measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis. E) Statistical differences on $G_{max}$ effects ($G_{max}$ fitted from the dose response curve) measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis.
3.7. PUFA analogues rescue LQT1 associated loss of function mutation

**Kv7.1 V215M + KCNE1 by left-shifting voltage dependence of $I_{Ks}$ activation.**

To evaluate the therapeutic potential of the PUFA analogues as potential treatments for LQTS, we expressed the $I_{Ks}$ channel bearing a mutation that causes Long QT Type 1 (LQT1) (V215M). V215M (in which a valine residue is replaced with methionine) is a loss-of-function mutation located in the S3 segment of the Kv7.1 α-subunit of the cardiac $I_{Ks}$ channel (Eldstrom et al. 2010). The V215M mutation causes a rightward shift in the voltage dependence of channel activation and alters the activation and deactivation kinetics compared to the wild type channel (Fig. 10A) (Eldstrom et al. 2010). In order to determine the ability of PUFA analogues to restore $I_{Ks}$ channel loss-of-function, we applied both lin-glycine and lin-taurine to the $I_{Ks}$ channel bearing the V215M mutation. From the current vs voltage relationship, we found that the V215M mutation results in a significant rightward shift in the voltage-dependence of $I_{Ks}$ channel activation relative to the wild type channel ($V_{0.5}$ of V215M = 40.2 ± 0.2 mV; Wild type $V_{0.5}$ = 16.6 ± 0.4 mV; $\Delta V_{0.5} = +24.2 \pm 2.3$ mV) (Fig. 10 B-C). However, the application of lin-glycine (at 10 μM) and lin-taurine (at 5 μM) both strongly left-shift the voltage-dependence of activation compared to the $V_{0.5}$ of V215M mutant channels ($\Delta V_{0.5}$ = -23.8 ± 3.3 mV and -29.7 ± 0.8 mV, respectively), fully restoring the wild-type voltage dependence of the $I_{Ks}$ channel (Fig. 10B-C). Lin-glycine and lin-taurine even shift the voltage-dependence to the left relative to the wild-type voltage dependence and increase the normalized tail current relative to wild-type (Fig. 10B). These data demonstrating PUFA-induced effects on LQT1-causing
mutations suggest that PUFA analogues are potent enough activators of the $I_{Ks}$ channel that they are capable of restoring the normal voltage dependence of LQT1 mutation-bearing $I_{Ks}$ channels.

Figure 3.12. PUFA analogues Lin-glycine and Lin-taurine rescue LQT1-associated loss-of-function mutation, V215M. A) B) Current-voltage relationship of the wild type $I_{Ks}$ channel (black squares; mean ± SEM; n = 4), Kv7.1 V215M + KCNE1 (red circles; mean ± SEM; n = 3), Kv7.1 V215M + KCNE1 with Lin-glycine (green triangles; mean ± SEM; n = 3), Kv7.1 V215M + KCNE1 with Lin-taurine (blue triangles; mean ± SEM; n = 3). C) Statistical differences on the voltage dependence ($V_{0.5}$) effects ($V_{0.5}$ fitted using the Boltzmann equation) measured by one-way ANOVA followed by Tukey’s HSD post hoc analysis.
General Discussion

We have characterized several different head groups of PUFA analogues in order to determine the range of effects of PUFA analogues on the current, voltage dependence, and maximal conductance of the cardiac \( I_{\text{Ks}} \) channel. Our findings demonstrate that PUFA analogues with a glycine head group consistently produce moderate activation of the cardiac \( I_{\text{Ks}} \) channel. In addition, we have demonstrated that PUFA analogues with a taurine or cysteic acid head groups produce the most potent activation of the cardiac \( I_{\text{Ks}} \) channel. Lastly, we have shown that increasing the number of potentially charged moieties did not necessarily improve PUFA-induced activation of the cardiac \( I_{\text{Ks}} \) channel. This is most likely due to the pKa of the additional potentially charged moieties, as well as potential steric hindrance of PUFAs with multiple potentially charged groups.

We have previously presented evidence that the charged head group of PUFAs electrostatically interact with S4 arginines or K326 in S6 the \( I_{\text{Ks}} \) channel (Liin, Yazdi, et al. 2018). We assume that the PUFAs and PUFA analogues tested here also interact by similar mechanisms with the \( I_{\text{Ks}} \) channel. As an example, we show here that neutralization mutations of charges in S4 or K326 in S6 decrease the effects of lin-glycine on the voltage shift and \( G_{\text{max}} \) (Suppl. Fig. S3), as if lin-glycine also interact with the S4 arginines and K326 in the pore.
Kv7.1 R231Q Q234R + KCNE1 + Lin-glycine

A

Normalized $I_{bat}$

Voltage (mV)

B

$V_0$

Concentration (μM)

C

Δ$V_{0.5}$ (mV)

Concentration (μM)

D

$G/G_{max}$

Concentration (μM)

Kv7.1 K326Q + KCNE1 + Lin-glycine

E

Normalized $I_{bat}$

Voltage (mV)

F

$V_0$

Concentration (μM)

G

Δ$V_{0.5}$ (mV)

Concentration (μM)

H

$G/G_{max}$

Concentration (μM)
Most of the variability in the effects of the different PUFA head groups on $I_{Ks}$ channels can be explained by the predicted pKa of the different head groups, which determines their protonation state in the membrane bound to the $I_{Ks}$ channel. Our group has previously demonstrated that the protonation state of the PUFA head group is influenced specifically by the presence of KCNE1 bound in the lipophilic cleft between adjacent Kv7.1 α-subunits (Larsson, Larsson, and Liin 2018). When KCNE1 is present, acidic residues located in the S5-P-helix loop (and in particular, the residue E290) are brought closer to the PUFA head group and, promoting protonation of the PUFA head group (Larsson, Larsson, and Liin 2018). When the pH dependence of $I_{Ks}$ activation is measured, the experimentally determined pH dependence a PUFA with a carboxyl head group was consistent with a pKa $\sim 8.5$, suggesting that PUFAs with a simple carboxyl head group is protonated and neutral at pH 7.5. We, therefore, propose that PUFAs with a simple carboxyl head group are unable to participate in an electrostatic interaction with S4 arginines or K326 in S6 the $I_{Ks}$ channel.
The experimental determined pKa value of PUFA analogues with a glycine head group associated with the I\textsubscript{Ks} channel is ~ 7.6 (Borjesson and Elinder 2011; Elinder and Liin 2017; Liin et al. 2015). Therefore half of the PUFA molecules with a glycine head group will be deprotonated and able to participate in an electrostatic interaction with S4 arginines or K326 in S6. (Liin et al. 2015). Our data comparing the effects of lin-glycine at pH 7.5 and pH 9 supports the idea that half of the PUFA molecules with a glycine head group are able to have an electrostatic interaction with the S4 segment of I\textsubscript{Ks} channels. Notably, the left-shift in the V\textsubscript{0.5} of lin-glycine at pH 9 (Fig. 3H) is approximately doubled compared to the left-shift at pH 7.5 (Fig. 3E), consistent with our estimate of a pKa of ~7.5-7.6 for PUFA analogues with glycine head groups (Suppl. Table 1). At pH 9, all of the lin-glycine molecules will be deprotonated and able to participate in an electrostatic interaction with the S4 segment, leading to a larger left-shift in the voltage-dependence of I\textsubscript{Ks} channel activation.

Finally, the predicted pKa for PUFA analogues with a taurine head group associated with I\textsubscript{Ks} channels is ~ 2.6, so that all taurine head groups are able to participate in an electrostatic interaction with the S4 arginines or K326 in S6 even at pH 7.5. The predicted pKas for taurine and glycine compounds are consistent with the approximate half size of the voltage-shifting effect of glycine compounds compared to taurine compounds. Why lin-glycine gives a larger increase in G\textsubscript{max} than lin-taurine is not clear, but maybe due to different access for lin-glycine than lin-taurine to the PUFA binding site that promotes increase in G\textsubscript{max}.
We estimated pKa$_1$ and pKa$_2$ values of lin-aspartate and lin-cysteic acid, as well as the pKa$_3$ of lin-AP3, to compare to estimated pKa values of lin-glycine and lin-taurine (Suppl. Table 1). In lin-aspartate, the pKa$_1$ is ~7.6, which is similar to the pKa value of lin-glycine, suggesting that the first potentially charged group is likely to reside in its deprotonated form 50% of the time at pH 7.5. The pKa$_2$ of lin-aspartate is ~9.1, which means that the second potentially charged moiety would be protonated and uncharged at pH 7.5. Therefore, lin-aspartate has approximately the same functional charge on the hydrophilic head group as lin-glycine. Indeed, the overall effect of lin-aspartate on $I/I_0$ is not significantly different than the $I/I_0$ effect of lin-glycine. In lin-cysteic acid, the pKa$_1$ is ~2.4, which is very similar to the pKa value of lin-taurine, meaning lin-cysteic acid should be at least as potent as lin-taurine. pKa$_2$ of lin-cysteic acid is ~7.1, meaning that the second group is likely to reside in its deprotonated form >50% of the time at physiological pH. However, lin-cysteic acid did not have a larger effect than lin-taurine, suggesting that the second charge group is not interacting with the channel or it is possible that nearby residues in the I$_{KS}$ channel protein modify the pKa$_2$ so that this group remains protonated at pH 7.5. Lastly, in lin-AP3, the pKa$_1$ and pKa$_2$ are ~5.0 and ~7.7 while the pKa$_3$ is ~11.8, which would suggest that the first site would be deprotonated and the second site would be deprotonated 50% of the time while the third group is protonated and uncharged at pH 7.5. However, Lin-AP3 has little to no effect on $I/I_0$, $\Delta V_{0.5}$, or $G_{max}$, suggesting that lin-AP3 does not effectively interact with the voltage sensor/pore
or it is not effectively deprotonated/negatively charged. We observe small effects of lin-AP3 when applied at pH 9 (in an attempt to help unmask potentially charged groups) (Suppl. Fig. S4), suggesting that there may be steric hindrance preventing the bulky AP3 head group from interacting favorably with the \( I_{Ks} \) channel.

**Figure 3.14. Effects on lin-AP3 on \( I_{Ks} \) activation at pH 9. A-C) Dose dependent effects of lin-AP3 on A) \( I_{Ks} \) current (\( I/I_0 \)), B) \( I_{Ks} \) voltage dependence (\( \Delta V_{0.5} \)), and C) \( I_{Ks} \) maximal conductance (\( G_{\text{max}} \)) at pH 9 (mean ± SEM at maximal concentration; \( n = 3 \)).**
Similar to our findings on the importance of the pKa of the PUFAs for shifting the voltage dependence of $I_{ks}$ channels, Ottosson and coworkers (2015) found that lowering the pKa of resin acid molecules resulted in greater left shift in the voltage dependence of the Shaker potassium channel (Ottosson et al. 2015). This further shows the importance of a deprotonated and charged compound for a strong activating effect on voltage-gated $K^+$ channels by the lipoelectric mechanism. In addition, Ottosson et al. noted that some substitutions wherein a bulky group was added to the scaffold, the efficacy of these resin acid compounds were reduced (Ottosson et al. 2015). They suggest that adding a bulky group may impede the ability of the small molecule to interact with the voltage sensor of the Shaker $K^+$ channel (Ottosson et al. 2015). This is similar to our data using the more bulky PUFA analogue lin-AP3.

The pH dependence of PUFA head group ionization has also been shown in the Slo1 BK channel by Tian and colleagues (2016) (Tian et al. 2016). They found that DHA produces potent activation of Slo1 BK channels and that this effect can be reduced when the pH is decreased, leading to protonation of the PUFA head group, and that the effect can be potentiated when the pH is increase, leading to deprotonation of the PUFA head group (Tian et al. 2016). Similar to our results, Tian et al. found that the addition of a phosphate head group leads to an attenuated effect on BK channel activation compared to DHA and DHA-glycine, which is similar to the effects we see when applying lin-AP3 (Tian et al. 2016).
In addition to the charge of the PUFA head group, the degree of unsaturation in the PUFA tail also plays an important role in PUFA-induced activation of the $I_{Ks}$ channel. We and others have found that the PUFA-induced activation of $I_{Ks}$ channels and Shaker K channels requires that the PUFA tail structure has at least two double bonds in *cis*-configuration in the tail (Liin et al. 2015; Liin et al. 2016) (Borjesson, Hammarstrom, and Elinder 2008). We recently conducted a systematic analysis of the PUFA tail (Bohannon et al. 2018) and found that neither the length of the carbon tail nor the number of double bonds in the tail correlated significantly with effects on or apparent binding affinity for the cardiac $I_{Ks}$ channel (Bohannon et al. 2018). However, the position of the double bonds in the tail is strongly correlated with stronger activation of and better apparent affinity for the cardiac $I_{Ks}$ channel (Bohannon et al. 2018).

Lipophilic compounds have the ability to form micelles. The concentration at which micelle formation takes place is called the critical micellar concentration (CMC). If the critical micellar concentration for our compounds is reached, micelle formation has the potential to interfere with the efficacy of the PUFAs and PUFA analogues being applied. Some factors that contribute to the ability of PUFAs to form micellar structures include the degree of unsaturation of the PUFA tail, the length of the PUFA tail (Borjesson, Hammarstrom, and Elinder 2008). In addition, the CMC could be influenced to some extend by pH (Rustan and Drevon 2005). However, the critical micellar concentration that is estimated for the majority of PUFAs and other unsaturated fatty acids is between 60-150
µM (Serth et al. 1991; Richieri, Ogata, and Kleinfeld 1992; Mukerjee and Mysels 1971). The experiments reported here were done at concentrations between 0.2-20 µM, which is well under the expected critical micellar concentration reported for unsaturated fatty acids. For this reason, we expect that the PUFAs applied in our preparation remain in the free fatty acid form, meaning that it is unlikely that any lack of effect from a PUFA could be attributed to the formation of micelles.

A range of effective compounds that activate the cardiac $I_{\text{KS}}$ channel is useful in the design of personalized therapeutics for LQT1. Patients with different LQT1 mutations have $I_{\text{KS}}$ channels with different degrees of channel malfunction (e.g. different size voltage shifts in their voltage dependence of activation) and present symptoms of varying severity. For this reason, individual LQT1 patients will not benefit from a one-size-fits-all treatment, producing a need for more personalized treatments. The findings presented here suggest that patients with more severe loss-of-function mutations of the cardiac $I_{\text{KS}}$ channel would most likely benefit from PUFA analogues with a taurine head group. In particular, PUFA analogues with a taurine or cysteic acid head group would be the most effective to rescue loss-of-function mutations in the $I_{\text{KS}}$ channel that lead to large shifts of the voltage dependence of $I_{\text{KS}}$ activation, because these head groups produce the most robust effects on the $V_{0.5}$. Patients with a milder LQT1 phenotype, however, may benefit more from treatment with a glycine PUFA analogue that has more moderate effects on $I_{\text{KS}}$ channel activation, and especially loss-of-function mutations that alter the maximal conductance ($G_{\text{max}}$) of the $I_{\text{KS}}$ channel. Effective
PUFA analogues can thus be selected for specific patients according to the severity of LQT1 pathology.
CHAPTER 4. Polyunsaturated fatty acid analogues have differential effects on cardiac Na\textsubscript{v}, Ca\textsubscript{v}, and K\textsubscript{v} channels through unique mechanisms\textsuperscript{3}

Background

The ventricular action potential is mediated by the coordinated activity of several different voltage-dependent ion channels (Mohrman and Heller 2010). The rapid upstroke of the ventricular action potential is mediated by the activation of the voltage-gated Na\textsuperscript{+} channel, Nav1.5, which then rapidly inactivates. The activation of L-type voltage gated Ca\textsuperscript{2+} channels, Cav1.2, and influx of Ca\textsuperscript{2+} leads to a sustained depolarization, or plateau phase, and the contraction of the cardiac muscle. Inactivation of Cav1.2 channels along with the activation of the slow delayed-rectifier K\textsuperscript{+} channels, Kv11.1 (which generates the $I_{Kr}$ current) and Kv7.1/KCNE1 (which generates the $I_{Ks}$ current), work to promote repolarization of the cell membrane (Nerbonne and Kass 2005). Mutations of any of these ion channels (or channelopathies) could lead to Long QT Syndrome (LQTS), which is an arrhythmogenic disorder that predisposes the individual to potentially fatal cardiac arrhythmias (Alders and Christiaans 2003; Bohnen et al. 2017).

The Nav1.5 α-subunit contains four non-identical linked domains, DI-DIV. Each of these domains contain 6 transmembrane segments (S1-S6), where the S1-S4

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segments make up the voltage-sensing domains (VSD) and S5-S6 segments make up the pore domains (PD). The S4 helix of each of the four domains contains a motif with positively charged amino acid residues which allow the S4 segment to detect and respond to changes in the membrane electric field, acting as the channel voltage sensor (Chanda and Bezanilla 2002). The movement of these voltage sensors determines the voltage dependence of activation and inactivation, where activation of the DI-III S4s are suggested to promote activation and activation of DIV S4 segment is sufficient to induce voltage-dependent inactivation of Nav1.5 (Capes et al. 2013). The Nav1.5 α-subunit exists as a macromolecular complex with the accessory subunit β1 (Barro-Soria, Liin, and Larsson 2017; Xiao et al. 2000; Zhu et al. 2017). Gain-of-function mutations of Nav1.5 increase Na⁺ currents and lead to LQTS Type 3 (LQT3) (Fernandez-Falgueras et al. 2017; Calloe et al. 2013; Rivolta et al. 2002).

Like Nav1.5, the Cav1.2 α-subunit contains four linked domains, DI-DIV, where each domain consists of 6 transmembrane segments S1-S6. S1-S4 form the VSD, where S4 acts as the voltage sensor, and S5-S6 form the PD. Cav1.2 exists as a large macromolecular complex with the accessory subunits β3 and α2δ subunits that are important for membrane expression and alter channel activation and deactivation kinetics, respectively (Rougier and Abriel 2016; Chen et al. 2004). Cav1.2 undergoes both voltage-dependent inactivation and calcium-dependent inactivation (Stotz, Jarvis, and Zamponi 2003; Zhang et al. 1994) which allows it to regulate Ca²⁺ influx into the cardiomyocyte. Gain-of-function
mutations of Cav1.2 increase Ca\textsuperscript{2+} currents and lead to Long QT Type 8 (LQT8) (Dick et al. 2016; Hoffman 1995).

The voltage-gated K\textsuperscript{+} channel, Kv7.1, along with the auxiliary subunit KCNE1, mediates an important repolarizing K\textsuperscript{+} current, I\textsubscript{Ks} (Noble and Tsien 1969a; Deal, England, and Tamkun 1996; Lei and Brown 1996). The Kv7.1 α-subunit contains 6 transmembrane segments, S1-S6. S1-S4 comprise the VSD, where S4 contains several positively charged amino acid residues that allow S4 to act as the voltage sensor of Kv7.1. S5-S6 segments comprise the channel PD. Kv7.1 forms a tetrameric channel, where 4 Kv7.1 α-subunits arrange to form a functional channel. The auxiliary β-subunit KCNE1 drastically modulates Kv7.1 channel voltage dependence, activation kinetics, and single-channel conductance (Barro-Soria et al. 2014; Osteen et al. 2010). Loss-of-function mutations in the Kv7.1 α-subunit and KCNE1 β-subunit lead to reductions in I\textsubscript{Ks} and can lead to LQTS Type 1 (LQT1) and Type 5 (LQT5) (Huang et al. 2018; Ma et al. 2015; Schwartz, Crotti, and Insolia 2012; Sanguinetti 1999; Harmer et al. 2009), respectively.

Polyunsaturated fatty acids (PUFAs) are amphipathic molecules that have been suggested to possess antiarrhythmic effects (Endo and Arita 2016; Kang and Leaf 2000). PUFAs, such as DHA and EPA, have been shown to prevent cardiac arrhythmias in animal models and cultured cardiomyocytes by inhibiting the activity of Na\textsubscript{v} and Ca\textsubscript{v} channels (Kang and Leaf 1996, 2000; Xiao 1997; Xiao et
al. 1995). Since the voltage sensors of Nav and Cav channels are relatively homologous, it has been suggested that PUFAs act on the voltage-sensing S4 segments that control inactivation in these channels (Kang and Leaf 1996, 2000). Our group has demonstrated that PUFAs and PUFA analogues also modulate the activity of the Kv7.1/KCNE1 channel and work to promote voltage-dependent activation of the $I_{Ks}$ current through a mechanism referred to as the lipoelectric hypothesis (Borjesson, Hammarstrom, and Elinder 2008; Liin et al. 2015; Liin et al. 2016). Our group has also demonstrated that PUFAs and modified PUFAs exert a second effect on the pore of Kv7.1 through an additional electrostatic interaction with a lysine residue (K326) in the S6 segment (Liin, Yazdi, et al. 2018). This electrostatic interaction between the negatively charged PUFA head group and K326 leads to an increase in maximal conductance of the channel ($G_{max}$) (Liin, Yazdi, et al. 2018).

Some groups have suggested that PUFAs could modify Nav channels by causing a leftward shift in voltage dependent inactivation through an electrostatic effect on the voltage-sensing domains involved in inactivation (Kang and Leaf 1996, 2000). It is possible that PUFAs modulate Kv7.1/KCNE1, Nav, and Cav channels by a similar mechanism by integrating next to the S4 voltage sensors and electrostatically attracting the voltage sensors toward their outward position. If PUFAs integrate preferentially next to the S4 that controls inactivation in Nav and Cav channels but next to all S4s in Kv7.1/KCNE1 channels, PUFAs would promote activation in Kv7.1/KCNE1 channels but promote inactivation in Nav and
Cav channels. Though both PUFAs and PUFA analogues are known to modulate different ion channel activities (i.e. processes underlying activation and inactivation), it is unclear whether specific PUFAs and PUFA analogues are selective for certain ion channels or if they broadly influence the activity of several different ion channels simultaneously.

In this work, we characterize the channel-specific effects of different PUFAs and PUFA analogues in order to further understand which PUFAs and PUFA analogues would be the most therapeutically relevant in the treatment for different LQTS subtypes. We have found that PUFA analogues modulate Kv7.1/KCNE1, Cav1.2, and Nav1.5 through different mechanisms instead of through a shared mechanism. In addition, we demonstrate that PUFA analogues exhibit a broad range of differences in selectivity for Kv7.1/KCNE1, Cav1.2, and Nav1.5. Lastly, PUFA analogues that are more selective for Kv7.1/KCNE1 are able restore a prolonged ventricular action potential and prevent arrhythmia in simulated cardiomyocytes.
Results

4.1. PUFA analogues modulate Kv7.1/KCNE1, Cav1.2, and Nav1.5 through distinct mechanisms.

There are several studies supporting electrostatic activation of Kv7.1/KCNE1 channels by PUFA analogues (Larsson, Larsson, and Liin 2018; Liin et al. 2015; Liin et al. 2016; Liin, Yazdi, et al. 2018). PUFAs are known to inhibit Na\textsubscript{v} and Ca\textsubscript{v} channels, but there is little evidence on the mechanism of channel inhibition using a diverse set of PUFA analogues. Previous groups have suggested that PUFAs may inhibit Na\textsubscript{v} and Ca\textsubscript{v} channels by interacting with S4 voltage sensors and stabilizing the inactivated state since there are similarities between the voltage sensor profiles of Na\textsubscript{v} and Ca\textsubscript{v} channels. (Xiao 1997; Kang and Leaf 1996, 2000; Xiao et al. 1995). For this reason, we hypothesize that PUFA analogues inhibit Cav1.2 and Nav1.5 through a shared electrostatic mechanism on S4 voltage sensors, similar to that reported with Kv7.1/KCNE1 channels. But in the case of Na\textsubscript{v} and Ca\textsubscript{v} channels, PUFAs would left-shift the voltage dependence of inactivation instead of activation which is seen in Kv7.1/KCNE1. To compare the effects of different PUFA analogues on these three different channels, we here measure the currents from Kv7.1/KCNE1, Cav1.2, and Nav1.5 expressed in Xenopus oocytes using two-electrode voltage clamp.

We first illustrate the effects of a representative PUFA analogue Linoleoyl taurine (Lin-taurine) on the voltage dependence of activation and the conductance of Kv7.1/KCNE1 (Fig. 1A-C). These effects are reflected in the tail current-voltage
relationship where the effects on the voltage sensor are measured as a leftward shift in the voltage dependence of activation and the effects on the conductance are measured as a relative increase in the maximal conductance upon PUFA application (Fig. 1C).

**Figure 4.1. PUFAs activate Kv7.1/KCNE1 channels through an electrostatic mechanism on voltage sensor and pore.**

A) Simplified membrane topology of a single Kv7.1 α-subunit (blue) and a single KCNE1 β-subunit (grey). B) Voltage protocol used to measure voltage dependence of activation and representative Kv7.1/KCNE1 current traces in control (0 μM) and 20 μM Lin-taurine. Arrows mark tail currents. C) Current-voltage relationship demonstrating PUFA induced left-shift in the voltage-dependence of activation ($V_{0.5}$) and increase in maximal conductance ($G_{max}$) (mean ± SEM; n = 3).
We also measure the effect of Lin-taurine on Cav1.2 and Nav1.5 channels (Figure 2-3). When we apply Lin-taurine to the Cav1.2 macromolecular complex (Fig. 2A), we see that Lin-taurine reduces Ca$^{2+}$ currents in a dose-dependent manner (Fig. 2B-C). However, Lin-taurine reduces Ca$^{2+}$ current without shifting the voltage-dependence of Cav1.2 activation (Fig. 2B-C; Supplemental Fig. 1). We use a depolarizing pre-pulse protocol to measure changes in voltage-dependent inactivation (Fig. 2D-E). When we measure the effects of PUFA analogues on voltage-dependent inactivation, we see again a decrease in Ca$^{2+}$ currents, but surprisingly no shift in voltage-dependent inactivation (Fig. 2D-E). This suggests that PUFA analogues do not inhibit Cav1.2 channels through a shared electrostatic mechanism on S4 voltage sensors that shifts the voltage dependence of S4 movement, but rather through a mechanism that reduces either the number of conducting channels (potentially through an effect on the pore) or the maximum conductance of each channel.
Figure 4.2. PUFAs inhibit Cav1.2 channels without altering channel voltage dependence. A) Simplified membrane topology of the Cav1.2 pore-forming α-subunit (light gray) and auxiliary β- (mint) and α2δ-subunits (yellow and green). B) Voltage protocol used to measure voltage dependence of activation and representative Cav1.2 current traces in control (0 μM) and 20 μM Lin-taurine. C) Current-voltage relationship demonstrating dose-dependent inhibition of Cav1.2 currents measured from activation protocol (mean ± SEM; n = 3). D) Voltage protocol used to measure voltage dependence of inactivation and representative Cav1.2 current traces in control (0 μM) and 20 μM Lin-taurine measured at arrow. E) Current-voltage relationship demonstrating dose-dependent inhibition of Cav1.2 currents measured from inactivation protocol (mean ± SEM; n = 3).
When we apply Lin-taurine to Nav1.5 (Figure 3A) and measure voltage-dependent activation, we see a dose-dependent inhibition of Na\(^+\) currents with no shift in the voltage dependence of activation (Fig. 3B-C; Supplemental Fig. 1). However, when we measured voltage-dependent inactivation of Nav1.5, we observed that PUFA analogues left-shift the voltage dependence of inactivation (Fig. 3D-E). In addition to the left-shift in voltage-dependent inactivation, we also observe a dose-dependent decrease in Nav1.5 currents (Fig. 3E). This suggests that PUFA analogues, while they do influence the voltage dependence of inactivation, may also have an additional effect on the conductance of Nav1.5, leading to the dose-dependent decrease in Na\(^+\) currents seen on top of the leftward shift of the voltage dependence of inactivation.
Figure 4.3. PUFAs inhibit Nav1.5 by shifting the voltage dependence of inactivation. A) Simplified membrane topology of the Nav1.5 pore-forming α-subunit (light blue) and auxiliary β-subunit (green). B) Voltage protocol used to measure voltage dependence of activation and representative Nav1.5 current traces in control (0 μM) and 20 μM Lin-taurine. C) Current-voltage relationship demonstrating dose-dependent inhibition of Nav1.5 currents measured from activation protocol (mean ± SEM; n = 5). D) Voltage protocol used to measure voltage dependence of inactivation and representative Nav1.5 current traces in control (0 μM) and 20 μM Lin-taurine measured at arrow. E) Current-voltage relationship demonstrating dose-dependent inhibition of Nav1.5 currents and leftward shift in the voltage dependence of inactivation measured from inactivation protocol (mean ± SEM; n = 5).
Figure 4.4. PUFA-induced changes in I/I₀ normalized by concentration show no changes in voltage-dependent activation of Cav1.2 and Nav1.5 channels. A-B) Voltage-dependent activation of Cav1.2 in the presence of A) N-AT and B) DHA-glycine. Peak currents are normalized to each concentration to clearly visualize that there is no shifts in voltage-dependent activation. C-D) Voltage-dependent activation of Nav1.5 in the presence of C) N-AT and D) DHA-glycine. Peak currents are normalized to each concentration to clearly visualize that there are no shifts in voltage-dependent activation.
Through these data, we observe that PUFA analogues modulate cardiac voltage-gated ion channels through non-identical mechanisms. PUFA analogues promote the activation of Kv7.1/KCNE1 through electrostatic effects that left-shift the voltage dependence of activation and an increase in the maximal conductance. PUFA analogues inhibit Cav1.2 channels through an apparent effect on the pore leading to a reduction in Ca\(^{2+}\) current but without producing any leftward shift in the voltage dependence of inactivation. In addition, PUFA analogues inhibit Nav1.5 through a combination of a leftward shift in the voltage dependence of inactivation and an effect on the maximum conductance, which leads to a dose-dependent decrease in Na\(^{+}\) current. Together these findings show that PUFA analogues affect Kv7.1/KCNE1, Nav1.5, and Cav1.2 channels through different mechanisms.

4.2. PUFA analogues with taurine head groups are non-selective and broadly modulate multiple cardiac ion channels, with preference for Cav1.2 and Nav1.5.

We have found through previous work that PUFA analogues with taurine head groups are good activators of the Kv7.1/KCNE1 channels due to the low pKa of the taurine head group (Liin et al. 2015; Liin et al. 2016). Having a lower pKa allows the taurine head group to be fully negatively charged at physiological pH so that it has maximal electrostatic effects on Kv7.1/KCNE1 channels (Liin et al. 2016). We tested a set of PUFA analogues with taurine head groups on Kv7.1/KCNE1, Cav1.2, and Nav1.5 channels to determine if these effects are
selective for the Kv7.1/KCNE1 channel or if taurine analogues also modulate Cav1.2 and Nav1.5 channels. Lin-taurine is a PUFA analogue with a taurine head group (Figure 4A) that promotes the activation of the cardiac Kv7.1/KCNE1 channel, by promoting a leftward shift in the voltage-dependence of activation by -39.9 ± 3.6 mV at 7 μM (p = 0.008) (Fig 4B). In addition, the application of Lin-taurine produces a slight, but not statistically significant increase in the maximal conductance of the Kv7.1/KCNE1 channel at 7 μM (1.9 ± 0.6; p = 0.26) (Fig. 4C). Lin-taurine inhibits Cav1.2 current in a dose-dependent manner without left-shifting the voltage dependence of inactivation for Cav1.2 (2.8 ± 1.4 mV; p = 0.17), but instead by significantly decreasing the G$_{\text{max}}$ at 7 μM (0.4 ± 0.1; p = 0.03) (Fig. 4D-E). Lastly, Lin-taurine inhibits Nav1.5 current by left-shifting the voltage dependence of inactivation (-23.5 ± 1.9 mV; p = 0.001) and also decreasing the G$_{\text{max}}$ at 7 μM (0.5 ± 0.07; p = 0.005) (Fig 4F-G).
Figure 4.5. Linoleoyl taurine has broad selectivity for Kv7.1/KCNE1, Cav1.2, and Nav1.5. A) Structure of Linoleoyl taurine (Lin-taurine). B, D, F) Dose response of the shift in voltage dependent B) activation (ΔV_{0.5}) of Kv7.1/KCNE1 channels (mean ± SEM; n = 3), D) inactivation (ΔV_{0.5}) of Cav1.2 channels (mean ± SEM; n = 3), F) inactivation (ΔV_{0.5}) of Nav1.5 channels (mean ± SEM; n = 5) in the presence of lin-taurine. C, E, G) Dose response of the change in maximal conductance (G_{max}) of C) Kv7.1/KCNE1 channels E) Cav1.2 channels, G) Nav1.5 channels in the presence of lin-taurine.
N-arachidonoyl taurine (N-AT) is a PUFA analogue with a taurine head group that has been demonstrated by our group to promote activation of Kv7.1/KCNE1, left-shifting the voltage-dependence and increasing the $G_{\text{max}}$ at 70 μM (Fig. 5A) (Liin et al. 2016). Here, we used lower concentrations (0.2, 0.7, 2, 7, and 20 μM) with the goal of understanding the selectivity of N-AT for cardiac ion channels and at more therapeutically feasible concentrations. Application of N-AT does not promote activation of Kv7.1/KCNE1 in this lower concentration range, does not left-shift of the voltage-dependence of activation (-1.8 ± 2.6 mV; p = 0.5), and does not increase the $G_{\text{max}}$ at 7 μM (0.9 ± 0.03; p = 0.98) (Fig. 5B-D). However, N-AT causes a dose-dependent decrease in Cav1.2 current, though does not cause a significant shift in the voltage dependence of inactivation (13.5 ± 3.8 mV; p = 0.07), nor does it cause a significant reduction in the overall $G_{\text{max}}$ (0.6 ± 0.1; p = 0.06) at 7 μM (Fig. 5E-G). In addition, N-AT decreases Nav1.5 current, produces a leftward shift in voltage-dependent inactivation (-16.7 ± 3.5 mV; p = 0.04), and significantly reduces the $G_{\text{max}}$ at 7 μM (0.3 ± 0.04; p = 0.004) (Fig. 5H-J). This data suggests that N-AT is more selective for Cav1.2 and Nav1.5, compared to Kv7.1/KCNE1.
Figure 4.6. N-arachidonoyl taurine is more selective for Cav1.2 and Nav1.5 than for Kv7.1/KCNE1. A) Structure of N-arachidonoyl taurine (N-AT). B) Current-voltage relationship of N-AT on Kv7.1/KCNE1 channels (mean ± SEM; n = 5). C) Dose response of the shift in voltage dependent activation (ΔV_{0.5}) of Kv7.1/KCNE1 channels in the presence of N-AT. D) Dose response of the change in maximal conductance (G_{max}) of Kv7.1/KCNE1 channels in the presence of N-AT. E) Current-voltage relationship of N-AT on Cav1.2 channels (mean ± SEM; n = 4). F) Dose response of the shift in voltage dependent inactivation (ΔV_{0.5}) of Cav1.2 channels in the presence of N-AT. G) Dose response of the change in maximal conductance (G_{max}) of Cav1.2 channels in the presence of N-AT. H) Current-voltage relationship of N-AT on Nav1.5 channels (mean ± SEM; n = 3). I) Dose response of the shift in voltage dependent inactivation (ΔV_{0.5}) of Nav1.5 channels in the presence of N-AT. J) Dose response of the change in maximal conductance (G_{max}) of Nav1.5 channels in the presence of N-AT.
Pinoleoyl taurine (Pin-taurine) promotes the activation of Kv7.1/KCNE1 in a dose-dependent manner (Fig. 6A-B). Pin-taurine, like Lin-taurine, promotes a leftward shift in the voltage dependence of activation (-23.8 ± 2.7 mV; p = 0.003) and increases the $G_{\text{max}}$ of Kv7.1/KCNE1 at 7 μM with a trend towards significance (2.2 ± 0.4; p = 0.06) (Fig. 6C-D). Pin-taurine also inhibits Cav1.2 current, but does not significantly shift the voltage-dependence of inactivation (4.1 ± 2.2 mV; p = 0.13) and does not significantly decrease the $G_{\text{max}}$ at 7 μM (0.8 ± 0.1; p = 0.2) (Fig. 6E-G). Pin-taurine inhibits Nav1.5 currents and does so by significantly left-shifting the voltage dependence of inactivation (-16 ± 2.7 mV; p = 0.01) and decreasing the $G_{\text{max}}$ at 7 μM (0.4 ± 0.09; p = 0.005) (Fig. 6H-J).
Figure 4.7. Pinoleoyl taurine has broad selectivity for Kv7.1/KCNE1, Cav1.2, and Nav1.5. A) Structure of Pinoleoyl taurine (Pin-taurine). B) Current-voltage relationship of pin-taurine on Kv7.1/KCNE1 channels (mean ± SEM; n = 4). C) Dose response of the shift in voltage dependent activation ($\Delta V_{0.5}$) of Kv7.1/KCNE1 channels in the presence of pin-taurine. D) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Kv7.1/KCNE1 channels in the presence of pin-taurine. E) Current-voltage relationship of pin-taurine on Cav1.2 channels (mean ± SEM; n = 5). F) Dose response of the shift in voltage dependent inactivation ($\Delta V_{0.5}$) of Cav1.2 channels in the presence of pin-taurine. G) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Cav1.2 channels in the presence of pin-taurine. H) Current-voltage relationship of pin-taurine on Nav1.5 channels (mean ± SEM; n = 4). I) Dose response of the shift in voltage dependent inactivation ($\Delta V_{0.5}$) of Nav1.5 channels in the presence of pin-taurine. J) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Nav1.5 channels in the presence of pin-taurine.
DHA-taurine promotes the activation of Kv7.1/KCNE1 channels in a dose-dependent manner, left-shifting the voltage-dependence of activation (-45.3 ± 2.9 mV; p = 0.004) and significantly increasing the $G_{\text{max}}$ at 7 μM (1.7 ± 0.1; p = 0.03) (Fig. 7A-C). DHA-taurine application results in dose-dependent inhibition of Cav1.2 current (Fig. 7E), but does not significantly left-shift the voltage-dependence of inactivation at 7 μM (0.2 ± 0.8 mV; p = 0.85) (Fig. 7F). Instead, DHA-taurine causes a significant decrease in the $G_{\text{max}}$ of Cav1.2 at 7 μM (0.4 ± 0.01; p < 0.001) (Fig. 7G). Lastly, DHA-taurine inhibits Nav1.5 by inducing a significant left-shift in the voltage-dependence of inactivation (-28.5 ± 0.6 mV; p < 0.001) and decreasing the $G_{\text{max}}$ at 7 μM (0.05 ± 0.01; p < 0.001) (Fig. 7H-I).

These results suggest that PUFA analogues with taurine head groups exhibit broad selectivity for multiple ion channels.
Figure 4.8. Docosahexanoyl-taurine has broad selectivity for Kv7.1/KCNE1, Cav1.2, and Nav1.5. A) Structure of docosahexanoyl taurine (DHA-taurine). B) Current-voltage relationship of DHA-taurine on Kv7.1/KCNE1 channels (mean ± SEM; n = 3). C) Dose response of the shift in voltage dependent activation (ΔV_{0.5}) of Kv7.1/KCNE1 channels in the presence of DHA-taurine. D) Dose response of the change in maximal conductance (G_{max}) of Kv7.1/KCNE1 channels in the presence of DHA-taurine. E) Current-voltage relationship of DHA-taurine on Cav1.2 channels (mean ± SEM; n = 3). F) Dose response of the shift in voltage dependent inactivation (ΔV_{0.5}) of Cav1.2 channels in the presence of DHA-taurine. G) Dose response of the change in maximal conductance (G_{max}) of Cav1.2 channels in the presence of DHA-taurine. H) Current-voltage relationship of DHA-taurine on Nav1.5 channels (mean ± SEM; n = 3). I) Dose response of the shift in voltage dependent inactivation (ΔV_{0.5}) of Nav1.5 channels in the presence of DHA-taurine. J) Dose response of the change in maximal conductance (G_{max}) of Nav1.5 channels in the presence of DHA-taurine.
4.3. PUFA analogues with glycine head groups tend to be more selective for Kv7.1/KCNE1 with lower affinity for Cav1.2 and Nav1.5.

PUFA analogues with glycine head groups have also been shown to effectively activate the Kv7.1/KCNE1 channel (Liin et al. 2015). The glycine head group has a lower pKa than regular PUFAs with a carboxyl head group (Liin et al. 2015), thereby allowing the head group to be more deprotonated and partially negatively charged at physiological pH. For this reason, PUFA analogues with glycine head groups are able to electrostatically activate Kv7.1/KCNE1 channels (Liin et al. 2015). We here tested several glycine compounds on Kv7.1/KCNE1, Cav1.2, and Nav1.5 channels to determine whether they have selective or non-selective effects on cardiac ion channels.

We first examined Linoleoyl glycine (Lin-glycine). Lin-glycine promotes the activation of the cardiac Kv7.1/KCNE1 channel by left-shifting the voltage dependence of channel activation to more negative voltages at 7 μM (-23.8 ± 1.6 mV; p < 0.001). Application of Lin-glycine also increases the Gmax of Kv7.1/KCNE1 at 7 μM (2.3 ± 0.2; p = 0.008) (Fig. 8A-D). Lin-glycine inhibits Cav1.2 in a dose-dependent manner (Fig. 8E), but does not left shift the voltage dependence of inactivation (0.6 ± 2.3 mV; p = 0.65). Instead, Lin-glycine produces a decrease of the Gmax at 7 μM, but this decrease is not statistically significant (0.3 ± 0.2; p = 0.07) (Fig. 8F-G). Lin-glycine causes a dose-dependent decrease of Nav1.5 current, left-shifts the voltage dependence of inactivation (-
15.2 ± 2.8 mV; p = 0.01), and reduces the $G_{\text{max}}$ at 7 μM (0.5 ± 0.1; p = 0.007) (Fig. 8H-J).

**Figure 4.9. Linoleoyl glycine has broad selectivity for Kv7.1/KCNE1, Cav1.2, and Nav1.5.**

A) Structure of Linoleoyl glycine (Lin-glycine).

B) Current-voltage relationship of lin-glycine on Kv7.1/KCNE1 channels (mean ± SEM; n = 4).

C) Dose response of the shift in voltage dependent activation ($\Delta V_{0.5}$) of Kv7.1/KCNE1 channels in the presence of lin-glycine.

D) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Kv7.1/KCNE1 channels in the presence of Lin-glycine.

E) Current-voltage relationship of lin-glycine on Cav1.2 channels (mean ± SEM; n = 4).

F) Dose response of the shift in voltage dependent inactivation ($\Delta V_{0.5}$) of Cav1.2 channels in the presence of lin-glycine.

G) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Cav1.2 channels in the presence of lin-glycine.

H) Current-voltage relationship of Lin-glycine on Nav1.5 channels (mean ± SEM; n = 4).

I) Dose response of the shift in voltage dependent inactivation ($\Delta V_{0.5}$) of Nav1.5 channels in the presence of lin-glycine.

J) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Nav1.5 channels in the presence of lin-glycine.
Pinoleoyl glycine (Pin-glycine) promotes the activation of Kv7.1/KCNE1 channels in a dose-dependent manner (Fig. 9A-B). Pin-glycine induces a left-shift in the voltage-dependence of activation (-8.7 ± 1.8 mV; p = 0.04) and increases the $G_{\text{max}}$ at 7 μM (1.7 ± 0.1; p = 0.03) (Fig. 9C-D). Pin-glycine, however causes little inhibition of Cav1.2 current (Fig. 9E). Pin-glycine produces no shift in the voltage dependence of inactivation (-3.1 ± 4.2 mV; p = 0.54) and does not significantly reduce the $G_{\text{max}}$ of Cav1.2 channels at 7 μM (0.8 ± 0.1; p = 0.34) (Fig. 9F-G). Pin-glycine inhibits Nav1.5 channels in a dose-dependent manner, but does not produce a significant left-shift in the voltage dependence of inactivation at 7 μM (-4.7 ± 1.9 mV; p = 0.09). However, there is a statistically significant reduction in the $G_{\text{max}}$ at 7 μM (0.7 ± 0.08; p = 0.03) (Fig. 9I-J).
Figure 4.10. Pinoleoyl glycine is more selective for Kv7.1/KCNE1 and Nav1.5 channels than for Cav1.2. A) Structure of Pinoleoyl glycine (Pin-glycine). B) Current-voltage relationship of pin-glycine on Kv7.1/KCNE1 channels (mean ± SEM; n = 3). C) Dose response of the shift in voltage dependent activation ($\Delta V_{0.5}$) of Kv7.1/KCNE1 channels in the presence of pin-glycine. D) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Kv7.1/KCNE1 channels in the presence of Pin-glycine. E) Current-voltage relationship of pin-glycine on Cav1.2 channels (mean ± SEM; n = 3). F) Dose response of the shift in voltage dependent inactivation ($\Delta V_{0.5}$) of Cav1.2 channels in the presence of pin-glycine. G) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Cav1.2 channels in the presence of Pin-glycine. H) Current-voltage relationship of pin-glycine on Nav1.5 channels (mean ± SEM; n = 4). I) Dose response of the shift in voltage dependent inactivation ($\Delta V_{0.5}$) of Nav1.5 channels in the presence of pin-glycine. J) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Nav1.5 channels in the presence of pin-glycine.
DHA-glycine promotes the dose-dependent activation of Kv7.1/KCNE1 channels (Fig. 10A-B), left-shifting the voltage-dependence of activation (-10.5 ± 1.0 mV; p < 0.002), and increasing the $G_{\text{max}}$ at 7 μM (1.9 ± 0.2; p = 0.03) (Fig. 10C-D).

However, DHA-glycine does not result in a dose-dependent decrease in Ca$^{2+}$ currents (Fig. 10E). DHA-glycine does not left-shift the voltage dependence of inactivation (7.6 ± 3.1 mV; p = 0.13) and does not significantly decrease the $G_{\text{max}}$ of Cav1.2 at 7 μM (1.0 ± 0.1; p = 0.98) (Fig. 10G). In addition, DHA-glycine produces some inhibition of Nav1.5, but only when applied at 20 μM (Fig. 10H).

While DHA-glycine produces a small, but significant left-shift in voltage dependent inactivation at 7 μM (-4.8 ± 1.9 mV; p = 0.01), it does not significantly reduce the $G_{\text{max}}$ at 7 μM (0.7 ± 0.08; p = 0.84) (Fig. 10H-J).
Figure 4.11. Docosahexanoyl glycine is more selective for Kv7.1/KCNE1 channels. A) Structure of docosahexanoyl glycine (DHA-glycine). B) Current-voltage relationship of DHA-glycine on Kv7.1/KCNE1 channels (mean ± SEM; n = 4). C) Dose response of the shift in voltage dependent activation ($\Delta V_{0.5}$) of Kv7.1/KCNE1 channels in the presence of DHA-glycine. D) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Kv7.1/KCNE1 channels in the presence of DHA-glycine. E) Current-voltage relationship of DHA-glycine on Cav1.2 channels (mean ± SEM; n = 3). F) Dose response of the shift in voltage dependent inactivation ($\Delta V_{0.5}$) of Cav1.2 channels in the presence of DHA-glycine. G) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Cav1.2 channels in the presence of DHA-glycine. H) Current-voltage relationship of DHA-glycine on Nav1.5 channels (mean ± SEM; n = 7). I) Dose response of the shift in voltage dependent inactivation ($\Delta V_{0.5}$) of Nav1.5 channels in the presence of DHA-glycine. J) Dose response of the change in maximal conductance ($G_{\text{max}}$) of Nav1.5 channels in the presence of DHA-glycine.
These results suggest that PUFA analogues with glycine head groups tend to be more selective for the cardiac Kv7.1/KCNE1 channel and tend to have lower apparent affinity for Cav1.2 and Nav1.5 channels. Pin-glycine and DHA-glycine both have more selective effects on the Kv7.1/KCNE1 channel and lower apparent affinity for Cav1.2 and Nav1.5 channels compared to PUFA analogues with taurine head groups. Lin-glycine, however, is less selective for Kv7.1/KCNE1 channels compared to Pin-glycine and DHA-glycine. Lin-glycine modulates Kv7.1/KCNE1 and Cav1.2 at similar concentrations, while the modulatory effects of Lin-glycine on Nav1.5 take place at higher concentrations. This suggests that the combination of a glycine head group and linoleic acid tail boosts the apparent affinity for Cav1.2 and Nav1.5 channels.

4.4. PUFA analogues with glycine head groups activate \( I_{KS} \) with higher apparent affinity compared to \( I_{Ca} \) and \( I_{Na} \).

We have observed that PUFA analogues have several different effects on the same channel (e.g. they alter voltage dependence and conductance at the same time). To evaluate the totally effects of PUFA analogues on channel currents at 0 mV (\( I/I_0 \)), we compared the dose response curves for \( I_{KS} \) (Kv7.1/KCNE1), \( I_{CaL} \) (Cav1.2), and \( I_{NaV} \) (Nav1.5) (Table 1). At 0 mV, 7 μM N-AT does not increase \( I_{KS} \) currents, but instead inhibits \( I_{CaL} \) currents and almost completely inhibits \( I_{NaV} \) currents (Fig. 11A; Table 1). By comparing the dose response curves and \( K_m \) (a measure of apparent binding affinity) for each channel current, we find that N-AT has similar apparent affinity for \( I_{KS} \), \( I_{CaL} \), and \( I_{NaV} \). At 7 μM, Lin-taurine increases
Ilks (though not significantly) while significantly inhibiting I_{CaL} and I_{NaV}, and exhibits higher apparent affinity for I_{CaL} and I_{NaV} than for I_{Ks} (Fig. 11B; Table 1). Similar to Lin-taurine, Pin-taurine and DHA-taurine increase I_{Ks} but it also inhibit I_{CaL} and I_{NaV} with higher apparent affinity for I_{CaL} and I_{NaV} than for I_{Ks} (Fig. 11C-D; Table 1). At 7 μM, Lin-glycine increases I_{Ks}, but also inhibits I_{CaL} and I_{NaV} with higher apparent affinity for I_{CaL} than for I_{Ks} and I_{NaV} (Fig. 11E; Table 1). At 7 μM, Pin-glycine increases I_{Ks}, but does not significantly inhibit I_{CaL} or I_{NaV}. When we compare the K_m from the dose response curves of each channel current, we find that Pin-glycine has higher apparent affinity for I_{Ks} compared to I_{NaV} (Fig. 11F; Table 1). Lastly, at 7 μM, DHA-glycine increases I_{Ks} with little effect on I_{CaL} and I_{NaV}, exhibiting higher apparent affinity for I_{Ks} and I_{CaL} than for I_{NaV} (Fig. 11G; Table 1). Overall, when we compare the effects of different PUFA analogues on I_{Ks}, I_{NaV}, and I_{CaL}, PUFA analogues with taurine head groups tend to have higher apparent affinity for I_{NaV} and I_{CaL}, whereas PUFA analogues with glycine head groups tend to have higher apparent affinity for I_{Ks}. 
Figure 4.12. Dose response curves for PUFAs on $I_{Ks}$, $I_{CaL}$, and $I_{Nav}$ at 0 mV. Dose response of A) N-AT, B) lin-taurine, C) pin-taurine, D) DHA-taurine, E) lin-glycine, F) pin-glycine, and G) DHA-glycine on $I_{Ks}$, $I_{CaL}$, and $I_{Nav}$ currents ($I/I_0$) at 0 mV.
<table>
<thead>
<tr>
<th>PUFA</th>
<th>$I_{KS}/I_0$ (at 7 μM) (mean ± SEM)</th>
<th>$K_m$ ($K_{KS}$) (μM) (mean ± SEM)</th>
<th>$I_{Ca}/I_0$ (at 7 μM) (mean ± SEM)</th>
<th>$K_m$ ($K_{Ca}$) (μM) (mean ± SEM)</th>
<th>$I_{Na}/I_0$ (at 7 μM) (mean ± SEM)</th>
<th>$K_m$ ($K_{Na}$) (μM) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-AT</td>
<td>1.12 ± 0.1 (p = 0.27)</td>
<td>9.8 ± 3.3</td>
<td>0.5 ± 0.1 (p = 0.04)</td>
<td>3.4 ± 2.5</td>
<td>0.2 ± 0.01 (p = 0.001)</td>
<td>3.1 ± 0.3</td>
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<tr>
<td>Lintaurine</td>
<td>7.7 ± 2.9 (p = 0.14)</td>
<td>11.4 ± 0.4</td>
<td>0.3 ± 0.1 (p = 0.02)</td>
<td>0.1 ± 0.01</td>
<td>0.1 ± 0.02 (p = 0.0001)</td>
<td>2.4 ± 0.04</td>
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<tr>
<td>Pintaurine</td>
<td>6.8 ± 1.0 (p = 0.01)</td>
<td>4.5 ± 0.2</td>
<td>0.7 ± 0.1 (p = 0.02)</td>
<td>NA</td>
<td>0.5 ± 0.1 (p = 0.01)</td>
<td>5.8 ± 1.7</td>
</tr>
<tr>
<td>DHA-taurine</td>
<td>5.1 ± 0.7 (p = 0.03)</td>
<td>5.9 ± 0.3</td>
<td>0.3 ± 0.05 (p = 0.02)</td>
<td>0.9 ± 0.6</td>
<td>0.07 ± 0.01 (p = 0.0001)</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Lingoicline</td>
<td>5.1 ± 0.4 (p = 0.002)</td>
<td>5.4 ± 0.2</td>
<td>0.4 ± 0.2 (p = 0.07)</td>
<td>2.2 ± 0.5</td>
<td>0.5 ± 0.1 (p = 0.02)</td>
<td>5.6 ± 0.5</td>
</tr>
<tr>
<td>Pingoicline</td>
<td>2.5 ± 0.2 (p = 0.02)</td>
<td>3.8 ± 0.4</td>
<td>0.8 ± 0.1 (p = 0.26)</td>
<td>NA</td>
<td>0.7 ± 0.1 (p = 0.09)</td>
<td>7.1 ± 1.2</td>
</tr>
<tr>
<td>DHA-glycine</td>
<td>3.7 ± 1.0 (p = 0.07)</td>
<td>9.4 ± 0.5</td>
<td>0.9 ± 0.1 (p = 0.19)</td>
<td>0.9 ± 0.03</td>
<td>1.1 ± 0.05 (p = 0.24)</td>
<td>16.7 ± 0.1</td>
</tr>
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</table>

$I/I_0$ represents the relative current of the specified channel. The $K_m$ indicates the concentration at which half the maximal effect on $I/I_0$ occurs and is used as a measure of the apparent affinity of the PUFA analogue. Data is represented at the mean ± SEM. Comparisons were made using One-way ANOVA and Student’s t-test. Significance level is set to $p = 0.05$. 
4.5. Selective Kv7.1/KCNE1 channel activators have antiarrhythmic effects in the simulated cardiomyocyte.

We next tried to understand what kind of compound is the most effective at shortening the action potential duration. To determine whether selective PUFA analogues or non-selective PUFA analogues can shorten the action potential duration, we simulated the effects of applying the PUFA analogues on human cardiomyocyte using the O’Hara-Rudy dynamic (ORd) model (O’Hara et al. 2011) while modifying parameters for the voltage dependence and conductance for individual channels to reflect our experimental PUFA-induced effects. We simulated the effects of PUFA analogues that are non-selective modulators for cardiac ion channels (i.e. N-AT, Lin-taurine, Pin-taurine, DHA-taurine, and Lin-glycine) at concentrations of 0.7 μM, 2 μM, and 7 μM (Fig. 12A-E). In most cases, we saw little change in the ventricular action potential until we reached 7 μM where we were unable to elicit an action potential (Fig. 12A-E). One exception was the effect of applying DHA-taurine, in which case we observed a small shortening of the action potential at 0.7 μM, but then an abnormal action potential upstroke and prolongation of the action potential at 2 μM (Fig 12D). This is likely due to the potent block of Nav1.5 channels, causing the action potential to be largely calcium dependent. But again, at 7 μM DHA-taurine, we were unable to elicit an action potential (Fig. 12D). However, the PUFA analogues that were more selective for Kv7.1/KCNE1, such as Pin-glycine and DHA-glycine (at 7 μM) induce a slight shortening of the wild type ventricular action potential (Fig. 12F-G). For Pin-glycine and DHA-glycine, we induced Long QT Type 2 by
simulating 25% block of the hERG channel, which generates the rapid
component of the delayed rectifier potassium current (I_{Kr}). 25% hERG block
prolongs the ventricular action potential by 50 ms. Application of Pin-glycine or
DHA-glycine at 7 \mu M in the simulation partially restores the duration of the
ventricular action potential. In addition to simulating the effects of PUFA
analogues on the ventricular action potential duration, we also simulated the
ability of 7 \mu M DHA-glycine (the most selective Kv7.1/KCNE1 activator) to
prevent arrhythmia by simulating early afterdepolarizations using 0.1 \mu M
dofetilide. Dofetilide is a blocker of the hERG channel and increases the
susceptibility for early afterdepolarizations (O'Hara et al. 2011) (Fig. 12H). When
we simulate 0.1 \mu M dofetilide + 7 \mu M DHA-glycine, we are able to suppress early
afterdepolarizations, suggesting that the application of 7 \mu M DHA-glycine would
be anti-arrhythmic (Fig. 12H).
General Discussion

We show here that PUFAs have different mechanisms of action on Kv7.1/KCNE1, Cav1.2, and Nav1.5 channels. We have previously shown that PUFAs promote the activation of Kv7.1/KCNE1 channels through the lipoelectric mechanism where the negatively charged PUFA head group electrostatically attracts both the S4 voltage sensor (facilitating its upward movement and channel opening) and K326 in S6 (increasing the maximal conductance) (Liin, Yazdi, et al. 2018; Borjesson and Elinder 2011; Borjesson, Hammarstrom, and Elinder 2008). In both Cav1.2 and Nav1.5 channels, PUFAs inhibit channel currents. We have found that PUFAs cause a dose-dependent reduction in the currents through in Cav1.2 channels, surprisingly with no effect on the voltage dependence of either activation or inactivation. In Nav1.5 channels, PUFAs cause inhibition through a dose-dependent decrease in currents, with both a left-shifting effect on the voltage dependence of inactivation and a decrease in conductance. We also demonstrate that PUFA analogues vary in their selectivity for voltage-gated ion channels. The selectivity depends on the specific concentration of PUFA applied, because several compounds have non-

Figure 4.13. PUFAs that are selective for Kv7.1/KCNE1 channels partially restore prolonged ventricular action potential and suppress early afterdepolarizations.
A-G) Simulated ventricular action potential in wild type cardiomyocytes (black) and in the presence of A) 0.7 (red), 2 (green), and 7 μM N-AT (blue), B) 0.7 (red), 2 (green), and 7 μM lin-taurine (blue), C) 0.7 (red), 2 (green), and 7 μM pin-taurine (blue), D) 0.7 (red), 2 (green), and 7 μM DHA-taurine (blue), E) 0.7 (red), 2 (green), and 7 μM lin-glycine (blue), F) 7 μM pin-glycine (blue solid), following 25% hERG block (red) and in the presence of 7 μM pin-glycine under 25% hERG block (blue dashed), and G) 7 μM DHA-glycine (blue solid), following 25% hERG block (red) and in the presence of 7 μM DHA-glycine under 25% hERG block (blue dashed). H) Early afterdepolarizations induced by dofetilide application (red) and suppression of early afterdepolarizations by 7 μM DHA-glycine in the presence of dofetilide (black).
overlapping dose response curves for their effects on the three different channels (Fig. 11). We also found that PUFAs with taurine head groups tend to have broad modulatory effects on Kv7.1/KCNE1, Nav1.5 and Cav1.2 channels, with higher apparent affinity for Cav1.2 and Nav1.5 channels. Conversely, PUFAs with glycine head groups tend to be more selective for Kv7.1/KCNE1 channels and display lower apparent affinity for Cav1.2 and Nav1.5 channels. By understanding the effects of PUFA analogues on individual channels, it opens up the possibility to target specific forms of LQTS in which specific channels are mutated.

In this work, we have demonstrated that PUFA analogues modulate several different voltage-gated ion channels, including those underlying the ventricular action potential: Kv7.1/KCNE1, Nav1.5, and Cav1.2. The effects of PUFAs on Kv7.1/KCNE1, Cav1.2, and Nav1.5 individually are anticipated to have anti-arrhythmic effects and would potentially be beneficial for patients with Long QT Syndrome. In the case of $I_{\text{Ks}}$ (Kv7.1/KCNE1) currents, PUFAs would be anti-arrhythmic by rescuing loss-of-function mutants of Kv7.1/KCNE1 ($I_{\text{Ks}}$) channels in Long QT Type 1 (KCNQ1 mutations) or 5 (KCNE1 mutations). In the case of $I_{\text{Na}}$ and $I_{\text{Ca}}$ currents, PUFAs would be anti-arrhythmic by inhibiting gain-of-function mutants of Nav1.5 and Cav1.2 channels in Long QT Type 3 or 7, respectively. We used the O’Hara-Rudy Dynamic model to simulate the ventricular action potential in the presence of different PUFAs. In our simulations, PUFAs that are non-selective (i.e. that activate Kv7.1/KCNE1 while inhibiting Cav1.2 and Nav1.5)
prevent the generation of an action potential. However, when we simulate the effects of Pin-glycine and DHA-glycine, which are both more selective for Kv7.1/KCNE1, we see a shortening in the action potential duration and the suppression of early afterdepolarizations. This suggests that selectively boosting \( I_{\text{KS}} \) (Kv7.1/KCNE1) current would be important for shortening and terminating the ventricular action potential. However, evaluating PUFA-induced effects on Kv7.1/KCNE1, Cav1.2, and Nav1.5 channels bearing LQTS-causing mutations would be the next step in understanding the therapeutic potential for PUFA analogues as treatments for different forms of LQTS.

In our experiments using PUFA analogues on Nav1.5, we observed both a shift in the voltage dependence of inactivation and a dose-dependent decrease in \( \text{Na}^+ \) currents. Extensive work has been done to characterize how each of the different voltage-sensing domains in Nav channels contribute to voltage-dependent activation and inactivation, many implicating DIV S4 in fast inactivation (Ahern et al. 2015; Capes et al. 2013). Recent work by Hsu and colleagues (2017) has also shown using voltage clamp fluorometry, the importance of both DIII and DIV in Nav channel inactivation (Hsu et al. 2017). Our data suggest that PUFAs may interact with S4 segments involved in voltage-dependent inactivation, allowing PUFA analogues to left-shift the voltage dependence of inactivation. However, this does not completely explain the additional dose-dependent decrease in \( \text{Na}^+ \) currents we observe on top of the leftward shifted voltage dependence of inactivation. Recent work by Nguyen and colleagues (2018) has uncovered a
mechanism of $Na_v$ channel inhibition through a new pathway, allowing a hydrophobic molecules to permeate a fenestration between domains III and IV (DIII and DIV) in the human cardiac Nav1.5 channel (Nguyen et al. 2019). It is possible that the hydrophobic PUFA analogue also block Nav1.5 channels through this fenestration between DIII and DIV, causing the voltage-independent decrease in sodium currents.

The molecular mechanism of action of PUFA analogues on Cav1.2 is still unclear, though we have shown that it does not occur through a shift in the voltage dependence of inactivation. In each case of Cav1.2 inhibition by PUFA analogues, we observe a dose-dependent decrease in the $Ca^{2+}$ currents that appears as a linear decrease in $I/I_0$ and $G_{\text{max}}$. There is evidence that some $Ca_v$ channel antagonists, such as dihydropyridines (DHPs) inhibit $Ca_v$ channels through an allosteric mechanism (Tang et al. 2016). Pepe and colleagues (1994) found that DHA alters the effectiveness of dihydropyridines, suggesting a shared binding site, or nearby binding sites, for DHPs and PUFAs (Pepe et al. 1994). Tang and colleagues (2016) found that dihydropyridines bind in a hydrophobic pocket near the pore of the bacterial $Ca_v$Ab channel and cause an allosteric conformational change that leads to disruption of the selectivity filter and thus inhibition of $Ca^{2+}$ currents (Tang et al. 2016). In addition, they observed that in the absence of DHPs a phospholipid occupies the DHP binding site (Tang et al. 2016). This would suggest that it is possible that PUFA analogues inhibit Cav1.2 by binding to, or near, the DHP binding site and causing an allosteric
conformational change that leads to a collapse of the pore and thus explaining
the inhibition of Cav1.2 currents without any changes in the voltage dependence
of inactivation.

Work from several groups has demonstrated a shared electrostatic mechanism of
action on voltage-gated K\textsuperscript{+} channels (Liin, Lund, et al. 2018) and voltage-gated
Na\textsuperscript{+} channels (Ahuja et al. 2015) by biaryl sulfonamides. Liin and colleagues
(2018) showed that biaryl sulfonamides promote the activation of the Shaker K\textsuperscript{+}
channel through an electrostatic effect on the voltage sensing domain. In addition,
Ahuja and colleagues (2015) showed that aryl sulfonamides inhibit Na\textsubscript{v} channels
through an electrostatic “voltage sensor trapping” mechanism that is specific for
the Nav1.7 isoform. The work by Ahuja et al. supports the ability to
pharmacologically target different ion channels with a high degree of selectivity
(Ahuja et al. 2015). This is in agreement with our findings using PUFA analogues
that show that PUFA analogues are variable in their channel selectivity, allowing
us to target particular ion channels involved in the ventricular action potential.

Our experiments were conducted using the *Xenopus laevis* oocyte expression
system, where voltage-clamp recordings were performed at room temperature. It
is possible that there may be temperature differences in the ways PUFA
analogues modify different ion channels that we are unable to capture by
conducting experiments at 20\textdegree C. There is also the possibility that the membrane
composition may differ between *Xenopus* oocytes and mammalian cells or
cardiomyocytes. However, using *Xenopus* oocytes, we are able to measure distinct differences between mechanisms of PUFA modulation in Kv7.1/KCNE1, Cav1.2, and Nav1.5 in isolation. To further confirm our findings, experiments should be conducted in mammalian cells or cardiomyocytes to determine the effects of different PUFA analogues on individual ion channels and the duration of the ventricular action potential at physiological temperatures.

The work presented here demonstrates that PUFA analogues exert diverse modulatory effects on different types of voltage-gated ion channels through non-identical mechanisms. Because PUFA analogues modulate Kv7.1/KCNE1 channels through electrostatic effects, we hypothesized they would have similar effects on Cav1.2 and Nav1.5 channels. However, our data suggests that PUFA analogues can exert various modulatory effects on the activity of different ion channels, and that the mechanism depends on the ion channel that is being modulated. In addition, we have shown that PUFA analogues exhibit a range of selectivity for different ion channels, which depends both on the PUFA head group and the combination of PUFA head and tail groups. Using simulations of the ventricular action potential, we have shown that selective Kv7.1/KCNE1 channel activators are the most effective at shortening a prolonged ventricular action potential and suppressing early afterdepolarizations induced by hERG block. This suggests that boosting Kv7.1/KCNE1 currents by using selective Kv7.1/KCNE1 channel activators can aid in restoring a normal action potential duration and possess antiarrhythmic potential.
CHAPTER 5. Discussion

The work presented here expands the current knowledge of interactions between polyunsaturated fatty acids and voltage-gated ion channels. PUFAs have been shown in the past to modulate the activity of voltage-gated ion channels including Nav, Cav, and Kv channels (Borjesson, Hammarstrom, and Elinder 2008; Elinder and Liin 2017; Kang and Leaf 1996; Xiao 1997; Liin et al. 2015; Liin et al. 2016). However, there has not been a systematic evaluation of the functional regions of the PUFA molecule (i.e. the head and tail groups of the PUFA) or the effects of the same PUFA on many voltage-gated ion channels expressed in a specific cell type. Here, I have presented systematic analysis of the role of PUFA tail group, head group, and the channel selectivity of different PUFAs.

Through this work, I have demonstrated that the PUFA tail group is important for the apparent binding affinity of the PUFA for the cardiac $I_{Ks}$ channel. I evaluated the length of the PUFA tail, the number of double bonds in the tail, and the position of the double bonds in the tail. Neither the length of the PUFA tail, nor the number of double bonds in the tail, significantly correlated with the effects on or the apparent binding affinity for the cardiac $I_{Ks}$ channel. However, the position of the double bonds in the tail relative to the PUFA head group does significantly correlate with the apparent binding affinity for the cardiac $I_{Ks}$ channel. Specifically, PUFAs that have double bonds closer to the PUFA head group promote the activation of the $I_{Ks}$ channel with higher apparent binding affinity. In addition, the position of the double bonds closer to the PUFA head group can be
used to predict effective and high affinity PUFAs that promote the activation of
the cardiac $I_{Ks}$ channel.

I have also demonstrated the importance of the $pK_a$ of the hydrophilic PUFA
head group in the activation of the cardiac $I_{Ks}$ channel, specifically that the $pK_a$ of
the head group is related to the magnitude of activation of the cardiac $I_{Ks}$
channel. In past studies, our group has observed that PUFAs with a carboxyl
head group, under physiological conditions (pH 7.5), are protonated and
uncharged when they are bound to the cardiac $I_{Ks}$ channel. This prevents them
from participating in an electrostatically activating the $I_{Ks}$ channel through
interactions with positively charged residues. However, when we apply PUFA
analogues with lower $pK_a$ head groups, we are again able to promote the
activation of the cardiac $I_{Ks}$ channel. This is because the lower $pK_a$ value of the
PUFA head group allows the PUFA to remain either partially or fully negatively
charged and participate in electrostatic interactions with the $I_{Ks}$ channel and
promote channel activation.

Lastly, I have demonstrated that PUFAs can have selective effects on voltage-
gated ion channels expressed in the heart. I looked at the effects of different
PUFAs on the cardiac Nav1.5, Cav1.2, and $I_{Ks}$ channels. Unlike the activating
effects of PUFAs on the $I_{Ks}$ channel, PUFAs inhibit Nav1.5 and Cav1.2 channels.
The results presented here demonstrate that PUFAs with a glycine head group
tend to have more selective effects on the $I_{Ks}$ channel. However, PUFAs that
have a taurine head group tend to have broad effects on Nav1.5, Cav1.2, and \( I_{Ks} \) channels. When the cumulative effects of the different PUFAs were simulated using the O’Hara-Rudy Dynamic, we observed that PUFAs that are more selective for the \( I_{Ks} \) channel can partially restore a prolonged ventricular action potential and can also prevent early afterdepolarizations that can lead to cardiac arrhythmia. On the other hand, PUFAs that have more broad effects on Nav1.5, Cav1.2, and \( I_{Ks} \) channels prevent the generation of the ventricular action potential in our simulations. This suggests that PUFAs have are more selective for the cardiac \( I_{Ks} \) channel may be more effective at preventing cardiac arrhythmia.

The causes of Long QT Syndrome involve mutations in various ion channels that are expressed in the heart. Patients with LQTS are generally treated using beta blockers to reduce adrenergic tone of the heart and prevent arrhythmia or through the surgical implantation of a cardioverter defibrillator. This means that the underlying ion channel dysfunction that leads to LQTS is not being addressed directly using traditional treatment methods. Polyunsaturated fatty acid analogues provide a means to directly modulate cardiac ion channels and potentially treat cardiac arrhythmia. We have observed in our own studies (and previous work) that PUFA analogues can restore loss-of-function of the cardiac \( I_{Ks} \) channel bearing LQTS-causing mutations (Liin et al. 2016). In addition, I have demonstrated in this work that PUFA analogues applied in solutions intended to mimic the normal physiological circulation of fatty acids in the body (ie. In combination with serum albumin and monounsaturated and saturated fatty acids)
are able to promote the activation of the cardiac $I_{Ks}$ channel by left-shifting the voltage-dependence of activation. These data further suggest that PUFA analogues have therapeutic potential for the treatment of cardiac arrhythmia in patients with LQTS.

The work presented here has provided a broad, systematic assessment of PUFA analogues and their effects on voltage-gated ion channels. By evaluating the functional role of the different structural regions of the PUFA, we have gained a foundation upon which new PUFA analogues could be synthesized. I have found that the position of the double bonds in the hydrophobic tail of the PUFA is important for the apparent binding affinity of PUFAs to the cardiac $I_{Ks}$ channel. With this information, it would be possible to make modifications to the PUFA tail in order to boost the apparent affinity for the $I_{Ks}$ channel. In addition, I found that the magnitude of PUFA-induced activation of the $I_{Ks}$ channel depends on a low $pK_a$ head group, allowing us to tune the potency of different PUFA analogues. Finally, I found that the selectivity of different PUFAs for cardiac ion channels (Nav1.5, Cav1.2, and Kv7.1/KCNE1 ($I_{Ks}$)) also depends on the PUFA head group. In this case, PUFA analogues with a glycine head group are more selective for the $I_{Ks}$ channels, whereas PUFA analogues with a taurine head group are more selective for Nav1.5 and Cav1.2 channels. With this information, it would be interesting to evaluate the role of the PUFA tail in apparent binding affinity of PUFAs to other voltage-gated ion channels, further promoting selective modulation of specific ion channels by altering the PUFA structure. So far, we
have looked only at the role of the hydrophobic tail in PUFAs that have a carboxyl head group, which we know is not negatively charged or effective at activating the I_{Ks} channel at physiological pH (7.5). An important next step would be to determine whether the double bonds in the PUFA tail have the same role in apparent binding affinity in PUFA analogues with glycine or taurine head groups (that are effective at activating the I_{Ks} channel at physiological pH). In addition, since the apparent affinity strongly influences the selectivity of different PUFA analogues for the voltage-gated ion channels expressed in the heart, it is important to know how the apparently affinity for Cav1.2 and Nav1.5 channels is influenced by the PUFA structure. In order to understand how the PUFA tail influences the apparent affinity of PUFA analogues for Cav1.2 and Nav1.5 channels, it is necessary to conduct a similar systematic analysis of the properties of the PUFA tail and how they correlate to the effects on and affinity for Cav1.2 and Nav1.5 channels. With this information, it would be possible to make modifications to newly synthesized PUFA analogues in order to tailor them to become more selective for specific cardiac voltage-gated ion channels.

One necessary step in the development of PUFA analogues as therapeutic compounds for LQTS is to screen new PUFA analogues against multiple ion channels. In this work, I have described that PUFA analogues that are more selective for the cardiac I_{Ks} channel are able to partially restore a prolonged ventricular action potential and prevent the initiation of early afterdepolarizations in simulated cardiomyocytes. However, PUFA analogues that activate the I_{Ks}
channel, but inhibit Nav1.5 and Cav1.2, prevent the initiation of the simulated ventricular action potential, likely due to extensive Nav1.5 channel block. This means that PUFA analogues should either be channel-selective or that the concentrations applied should maximize activation of the \( I_{Ks} \) channel while minimizing Nav1.5 block so that there are no disruptions of action potential initiation or conduction velocity. Another critical repolarizing current in the ventricular action potential is mediated by the \( I_{Kr} \) channel. However, PUFAs do not alter \( I_{Kr} \) and \( I_{K1} \) currents (unpublished data). In this preparation, the Kv11.1 channel was expressed alone, without the auxiliary subunit KCNE2 which associates with Kv11.1. A critical step in understanding how PUFA analogues alter action potential repolarization is to test newly developed PUFA analogues on Kv11.1/KCNE2 channels in order to fully characterize the selectivity of PUFA analogues.

In this work, I have shown that PUFA analogues can exert their modulatory effects on voltage-gated ion channels through a variety of mechanisms. Despite the similar architecture of Kv, Nav, and Cav channels, PUFA analogues modulate each of these channels through unique mechanisms rather than through a common, shared electrostatic mechanism. We have seen that PUFA analogues inhibit Nav1.5 by left-shifting the voltage dependence of inactivation, on top of a reduction in \( \text{Na}^+ \) currents. Preliminary data from our group has shown that mutations of the gating charges in the S4 segment of domain IV (DIV) in Nav1.5 removes part of the voltage-shifting effect of PUFA analogues. However,
the mutation of these residues does not completely abolish the left-ward shift in the voltage dependence of inactivation induced by PUFA analogues. There is evidence that the DIII S4 is also involved in voltage dependent inactivation of Nav1.5 (Ahern et al. 2015; Capes et al. 2013; Muroi et al. 2010). Future studies should include the mutagenesis of gating charge residues in DIII-DIV S4 segments in order to identify potential PUFA binding sites. Recent work has also shown that there is a lipophilic cleft in Nav channels that hydrophobic compounds can penetrate and exert modulatory effects. It is possible that hydrophobic PUFA analogues are able to bind in this lipophilic cleft and thus alter channel conductance, explaining the additional decrease in current observed in Nav1.5 block by PUFAs (Gamal El-Din et al. 2018). In Cav1.2 channels, we did not observe any voltage-shifting effects of the PUFA analogues tested, rather we observe a dose-dependent decrease in current. There is evidence using dihydropyridines (DHPs), that there is an allosteric mechanism if Cav channel block in which one DHP molecule binds and the subsequent conformational change disrupts the symmetry of the selectivity filter, leading to Cav channel block (Tang et al. 2016). There is also evidence that DHPs and PUFAs share a common binding side in the cardiac Cav channel (Pepe et al. 1994). It is possible that PUFAs modulate Cav1.2 by a similar allosteric mechanism as DHPs. In order to determine whether our PUFA analogues inhibit Cav1.2 by a similar mechanism as DHPs, it would be necessary to conduct experiments applying both DHPs and PUFA analogues to determine if these compounds compete for
the same binding site of the Cav1.2 channel, providing further insight into the mechanism of PUFA-induced block of Cav1.2 channels.

It is important to address that there are critical differences that could occur between the application of PUFA analogues to ion channels expressed in *Xenopus laevis* oocytes and the channels found in the human cardiomyocyte. For example, there may be differences in the phospholipid constituents of the membrane that could alter the incorporation of and/or the effects of exogenously applied PUFA analogues. In addition, many ion channels undergo posttranslational modification, including glycosylation. Glycosylation can have many effects on voltage-gated ion channels, including alterations to channel gating behavior, channel trafficking, and potential disruptions of interactions with small molecules/ligands and with other proteins. For example, Glycosylation is important for the trafficking of the b2 subunit associated with Nav1.5 channels to the cell membrane, which thus effects membrane expression of Nav1.5 (Cortada, Brugada, and Verges 2019). In addition, glycosylation has been shown to alter the gating kinetics of the Shaker Kv channel (Lopez-Rodriquez and Holmgren 2018). There is evidence that even exogenously expressed proteins can undergo posttranslational modifications, even when expressed in *Xenopus laevis* oocytes (Colman, Bhamra, and Valle 1984). However, in order for PUFA analogues to move forward as potential therapeutics for the treatment of LQTS, it is critical for them to be tested using mammalian systems to fully understand the potential of PUFA analogues when applied to the human body. To study the
effects of new PUFA analogues on different ion channels in a lipid environment specific to mammalian systems, ion channels can be expressed in a mammalian heterologous expression system such as HEK293 cells. Doing so will provide both information regarding the mechanism of action for different PUFA analogues on voltage-gated ion channels in a context that more accurately reflects the membrane composition and can also recapitulate physiological temperatures (37°C).

Given that PUFAs have an ionizable, hydrophilic head group and a long hydrophobic, hydrocarbon tail they have the ability to behave as detergents. It is, therefore, possible that PUFAs could form micelles which could lead to changes in the properties of the lipid bilayer. It is also possible for exogenously applied PUFAs to cause changes in membrane elasticity that modulate the activity of membrane proteins, including ion channels (Lee 2006). For example, the length of the fatty acid acyl chain that incorporates into the membrane can lead to changes in the thickness of the membrane, which could indirectly alter ion channel activity (Lee 2006). For example, the PUFA DHA is able to modulate the activity of gramicidin channels via changes in membrane fluidity (Bruno, Koepe III, and Andersen 2007). One basis for the membrane fluidity hypothesis is that PUFAs alter the activity of different ion channels at similar concentrations suggesting a similar mechanism (Bruno, Koepe III, and Andersen 2007). However, in this work, I found significant differences in apparent binding affinity for different voltage-gated ion channels expressed in the heart and can modulate
some channels selectively while having little to no effect on other cardiac ion channels. These data suggest that it is likely a direct effect on voltage-gated ion channel activity rather than an indirect effect through changes in membrane fluidity.

PUFAs are fatty acids that must be obtained through the diet. Linoleic acid is the precursor molecule of arachidonic acids, whereas $\alpha$-linolenic acid is the precursor molecule of DHA and EPA (Anderson and Ma 2009). Linoleic acid and $\alpha$-linolenic acid are found in vegetable oils, and the dietary sources of arachidonic acid, DHA, or EPA are fats (in the case of arachidonic acid) and fish oil (in the cases of DHA and EPA) (Anderson and Ma 2009). PUFAs (namely arachidonic acid and EPA) can be metabolized to molecules known as eicosanoids, a family of compounds involved in inflammation that include leukotrienes, thromboxanes, and prostaglandins (Anderson and Ma 2009). It is unclear, however, whether PUFA analogues (such as those tested in this work) would be further broken down into active molecules similar to the eicosanoid byproducts of EPA and arachidonic acid. Many of the PUFA analogues tested in the latter chapters of this work differ from EPA and arachidonic acid in that they have different chemical moieties on the PUFA head group and also have different tail structures (i.e different lengths, number of double bonds, and position of double bonds). It is possible that PUFA analogues could be broken down in the body, however there would likely be dissimilarities between the metabolic byproducts of PUFAs compared to those of PUFA analogues. To
understand the ramifications of long-term application of PUFA analogues, and the potential for the production of active PUFA analogue metabolites, PUFA analogues could be applied chronically in hiPSC-derived cardiomyocytes.

In addition, it is important understand the effects of PUFA analogues specifically on cardiomyocytes and the ventricular action potential. To do this, PUFA analogues can be applied first to human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). The duration of the ventricular action potential can be measured either using optical dyes (voltage sensing or calcium sensing) or using whole-cell patch-clamp electrophysiology. In LQTS, the ventricular action potential is prolonged, correlating to a prolongation of the QT interval measured by the electrocardiogram. The goal of finding PUFA analogues as potential therapeutics for LQTS is to find PUFA analogues that shorten the duration of the ventricular action potential. To determine if PUFA analogues can shorten a prolonged ventricular action potential, one could either prolong the ventricular action potential pharmacologically (using blockers such as chromonol or E4031) and apply PUFA analogues. Using hiPSC-CMs it is also possible to use lines of cardiomyocytes derived from LQTS patient cells and measure the effects of PUFA application on a prolonged ventricular action potential caused by and LQTS-causing mutation.

Eventually, PUFA analogues will need to be tested using animal models. Zebrafish offer a simple animal model where PUFA analogues can be tested on
the isolated zebrafish heart. The zebrafish heart can be dissected out and the action potential duration measured using optical dyes. Zebrafish also allow simple genetic manipulation which can be used to generate lines of zebrafish harboring LQTS-causing mutations (Leong et al. 2010). Additionally, in previous work, our group has applied PUFA analogues to whole guinea pig hearts following pharmacological prolongation of the ventricular action potential (Liin et al. 2015). Once strong candidates of PUFA analogues are determined, these compounds can be tested in isolated guinea pig hearts to probe further their therapeutic relevance in animal models. Eventually, there target compounds need to be tested in intact animals to determine their therapeutic potential \textit{in vivo}. The ideal route of drug administration of PUFA analogues for the treatment of LQTS in humans, would be oral administration. Therefore, the effects of PUFA analogues \textit{in vivo} could be tested using a supplemented food source given to animals harboring LQTS-causing mutations (such as zebrafish, guinea pigs, or rabbits).

Overall, the work presented here demonstrates that PUFA analogues offer promising pharmacological candidates for the treatment of Long QT Syndrome and cardiac arrhythmia.
CHAPTER 6. Materials and Methods

Molecular biology
cRNA encoding Kv7.1, KCNE1, Nav1.5 and β1, and Cav1.2, β3, and α2δ were transcribed using the mMessage mMachine T7 kit (Ambion). 50 ng of cRNA was injected into defolliculated *Xenopus laevis* oocytes (Ecocyte, Austin, TX): For \( I_{Ks} \) channel expression, we injected a 3:1, weight:weight (Kv7.1:KCNE1) cRNA ratio. For Nav1.5 channel expression, we injected a 2:1, weight:weight (Nav1.5:β1) cRNA ratio. For Cav1.2 channel expression, we injected a 2:1:1, weight:weight (Cav1.2:β3:α2δ) cRNA ratio. Site-directed mutagenesis was performed using the Quickchange II XL Mutagenesis Kit (QIAGEN Sciences, Maryland, USA) for mutations in the Kv7.1 \( \alpha \)-subunit. Injected cells were incubated for 72-96 hours in standard ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 5 mM HEPES; pH = 7.5) containing 1 mM pyruvate at 16ºC prior to electrophysiological recordings.

Two-electrode voltage clamp (TEVC)
*Xenopus laevis* oocytes were recorded in the two-electrode voltage clamp (TEVC) configuration. Recording pipettes were filled with 3 M KCl. The recording chamber was filled with ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 5 mM HEPES; pH 7.5). For Cav1.2 channel recordings, *Xenopus* oocytes were injected with 50 nl of 100 mM EGTA and incubated at 10ºC for 30 minutes prior to electrophysiological recordings in order to sequester cytosolic calcium. In addition, Cav1.2 channel recordings were done in solutions lacking Ca\(^{2+}\), using Ba\(^{2+}\) as the charge carrier to prevent calcium-dependent inactivation of Cav1.2.
channels. PUFAs were obtained from Cayman Chemical (Ann Arbor, MI.) or synthesized in house through methods previously described (Linköping, Sweden) and kept at -20°C as 100 mM stock solutions in ethanol. Serial dilutions of the different PUFAs were prepared from stocks to make 0.2 μM, 0.7 μM, 2 μM, 7 μM, and 20 μM concentrations in ND96 solutions (pH = 7.5). PUFAs were perfused into the recording chamber using the Rainin Dynamax Peristaltic Pump (Model RP-1) (Rainin Instrument Co., Oakland, CA. USA).

Electrophysiological recordings were obtained using Clampex 10.3 software (Axon, pClamp, Molecular Devices). To measure Kv7.1/KCNE1 currents we apply PUFAs as the membrane potential is stepped every 30 sec from -80 mV to 0 mV for 5 seconds before stepping to -40 mV and back to -80 mV to ensure that the PUFA effects on the current at 0 mV reached steady state. A voltage-step protocol was used to measure the current vs. voltage (I-V) relationship before PUFA application and after the PUFA effects had reached steady state for each concentration of PUFA. Cells were held at -80 mV followed by a hyperpolarizing prepulse to -140 mV. The voltage was then stepped from -100 to 60 mV (in 20 mV steps) followed by a subsequent voltage step to -20 mV to measure tail currents before returning to the -80 mV holding potential. For Cav1.2 channel recordings, PUFAs are applied as the membrane potential is stepped from -80 mV to -30 mV and then 10 mV before returning to the holding potential of -80 mV. This allows the PUFA effects to reach steady state before recording voltage dependent activation and inactivation. To measure voltage-dependent activation
of Cav1.2, cells are held again at -80 mV and then stepped from -70 mV to 40 mV (in 10 mV steps). Voltage-dependent inactivation was measured by holding cells at -80 mV, applying a 500-ms conditioning prepulse at voltages between -80 mV and 20 mV (in 10 mV steps) before stepping to a test pulse of 10 mV to measure the remaining current and returning to -80 mV holding potential. For Nav1.5 channel recordings, PUFAs are applied as the membrane potential is stepped from -80 mV to -90 mV for 480 ms before stepping to 30 mV for 50 ms and returned to a holding potential of -80 mV. This allows the PUFA effects to reach steady state before recording voltage-dependent activation and inactivation. To measure voltage-dependent activation of Nav1.5, cells are held at -80 mV and then stepped from -90 mV to 40 mV (in 10 mV steps) and then returning to -80 mV holding potential. Voltage-dependent inactivation was measured by holding cells at -80 mV, applying a 500-ms conditioning prepulse at voltages between -140 mV and -30 mV (in 10 mV steps) and measuring the remaining current at a test pulse of -30 mV before returning to -80 mV holding potential.
Data analysis

Tail currents from Kv7.1/KCNE1 measures were analyzed using Clampfit 10.3 software in order to obtain conductance vs. voltage (G-V) curves. The $V_{0.5}$, the voltage at which half the maximal current occurs, was obtained by fitting the G-V curves from each concentration of PUFA with a Boltzmann equation:

$$G(V) = \frac{G_{\text{max}}}{1 + e^{(V - V_{1/2})/s}}$$

where $G_{\text{max}}$ is the maximal conductance at positive voltages and $s$ is the slope factor in mV. The current values for each concentration at 0 mV ($I/I_0$) were used to plot the dose response curves for each PUFA. These dose response curves were fit using the Hill equation in order to obtain the $K_m$ value for each PUFA:

$$\frac{I}{I_0} = 1 + \frac{A}{1 + \frac{K_m^n}{x^n}}$$

where $A$ is the fold increase in the current caused by the PUFA at saturating concentrations, $K_m$ is the apparent affinity of the PUFA, and $n$ is the Hill coefficient. The maximum conductance ($G_{\text{max}}$) was calculated by taking the difference between the maximum and minimum current values (using the G-V curve for each concentration) and then normalizing to control solution (0 μM). In Cav1.2 and Nav1.5 channels, peak currents (normalized to the peak values in control ND96) were used to determine PUFA induced changes in $I/I_0$, $\Delta V_{0.5}$ of inactivation, and $G_{\text{max}}$. Graphs plotting $I/I_0$, $\Delta V_{0.5}$, $G_{\text{max}}$, and $K_m$ were generated using the Origin 9 software (Northampton, MA.).
Simulations

The effects of individual PUFA analogues were simulated on each ion channel using Berkeley Madonna modeling software and equations from the MATLAB code in the O'Hara and Rudy Dynamic (ORd) model (O'Hara et al. 2011). We individually simulated the Kv7.1/KCNE1, Cav1.2, and Nav1.5 channels in Madonna and altered the parameters suggested to be modulated by PUFA binding to recapitulate our voltage clamp data from Xenopus oocytes. For example, to model the effects observed on the cardiac \( I_{\text{Ks}} \) channel, we modified the voltage dependence of channel activation by shifting the \( V_{0.5} \) as well as multiplying the \( I_{\text{Ks}} \) conductance by the factor increase we observed in our experiments at a given PUFA concentration.

MATLAB simulations of the ventricular action potential in the epicardium of the heart were performed using the ORd model (O'Hara et al. 2011). To simulate the effects of PUFAs, we introduced the same modified parameters in the MATLAB code as we used to model the PUFA effects on the ionic currents in Berkeley Madonna. We made simultaneous changes to Kv7.1/KCNE1, Cav1.2, and Nav1.5 for a given PUFA and specific PUFA concentration to model the effects of different PUFA analogues on the ventricular action potential under wild type and LQTS conditions. To simulate susceptibility to early afterdepolarizations, hERG block by 0.1 \( \mu \text{M} \) dofetilide was simulated which previously has been shown to cause spontaneous early afterdepolarizations (O'Hara et al. 2011). To simulate the ability of PUFA analogues to suppress early afterdepolarizations, we altered
the activity of Kv7.1/KCNE1, Cav1.2, and Nav1.5 channels according to the
PUFA-induced effects observed during experiments.

*Estimated pK\(_a\) values*

pK\(_a\) for each of the PUFA head groups are calculated using the structure-based
Marvin Software (ChemAxon). However, studies of PUFAs in lipid bilayers and
our previous studies on PUFA-I\(_{\text{ks}}\) channel interactions have shown that there is a
large difference in the pK\(_a\) values in solution (calculated according to the
structure) compared to the measured pK\(_a\) of PUFAs in the lipid bilayer (Elinder
and Liin 2017; Borjesson and Elinder 2011) and in close contact with the I\(_{\text{ks}}\)
channel (Elinder and Liin 2017; Liin et al. 2015). The average difference between
the calculated pK\(_a\) values (of PUFA in solution) and experimentally determined
pK\(_a\) values for PUFAs associated with the I\(_{\text{ks}}\) channel is ~3.5 (82). We therefore
add this value (3.5) as a correction factor to the calculated solution pK\(_a\) values to
generate our estimated pK\(_a\) value for PUFAs associated with the I\(_{\text{ks}}\) channel. It is
this estimated pK\(_a\) value (calculated solution pK\(_a\) from Marvin Software + 3.5)
that is reported throughout this work. For example, the starting pK\(_a\) value for
linoleic acid in solution, generated using the Marvin Software is 5.0. This
calculated pK\(_a\) value (5.0) + the correction factor (3.5) is equal to 8.5, leading us
to report the estimated pK\(_a\) value for linoleic acid as 8.5.
Hierarchical Cluster Analysis

Hierarchical cluster analysis was performed using either R-based software available from wessa.net or using Biovinci data visualization software (Biovinci, Bioturing). Effects on $I/I_0$, $\Delta V_{0.5}$, and $G_{\text{max}}$ were normalized to the PUFA analogue with the largest influence on each of the three effects so that these effects were now scaled from 0.0 – 1.0, 1.0 being the largest effect. Each parameter ($I/I_0$, $\Delta V_{0.5}$, and $G_{\text{max}}$) was then used as input for clustering to generate the dendrogram and heat map. The dendrogram displays groupings of PUFAs and PUFA analogues according to similarity of their effects. The heat map displays the magnitude of the effects, with warmer colors representing PUFAs and PUFA analogues that have larger relative effects (closer to 1.0) on $I/I_0$, $G_{\text{max}}$, and $\Delta V_{0.5}$ and cooler colors representing PUFAs and PUFA analogues with smaller relative effects (closer to 0.0).

Statistics

Single and multivariable regression statistics were computed using GraphPad Prism (GraphPad Software, La Jolla, CA) and Origin 9 software, respectively. We conducted single linear regression on each of the PUFA parameters with each of the effects we were interested in (i.e. $I/I_0$, $V_{0.5}$, $G_{\text{max}}$, and $K_m$) to determine the slope of the fit, the adjusted $R^2$, and t-values. In addition, we conducted multivariable regression for all of the PUFA parameters simultaneously with each of the effects to determine again the slope, the adjusted $R^2$, and F-values. To determine if there were significant differences between PUFA-induced effects on $I/I_0$, $\Delta V_{0.5}$, and $G_{\text{max}}$ we conducted One-way ANOVA followed by Tukey’s HSD for
multiple comparisons. Significance α-level is set at p < 0.05 – asterisks denote significance: p < 0.05*, p < 0.01**, p < 0.001***.

**Multivariable regression analysis**

To test whether the PUFA effects – I/I₀, ΔV₀.₅, Gₘₐₓ, and Kₘ of I/I₀ – depend on a combination of the different parameters (Length, number of double bonds, and location of the first and last double bonds) of the PUFA tails, we conducted a multivariable regression of each PUFA effect using combinations of the four PUFA parameters simultaneously. The multivariable regression using all four parameters improved the fit for I/I₀ (Adjusted R² increased from 0.40 to 0.54) and Gₘₐₓ (Adjusted R² increased from 0.25 to 0.60). According to the multivariable regression analysis I/I₀ is significantly correlated to the location of the first double bond (Table 2). Gₘₐₓ is significantly correlated to the length of the tail, the number of double bonds and the location of the first and last double bond (Table 2). However, the ΔV₀.₅ and Kₘ (for I/I₀) single regressions were not improved by the addition of more parameters of the PUFA. This suggests that the ΔV₀.₅ and Kₘ (for I/I₀) are highly dependent on single PUFA parameters (i.e. the position of the last double bond and first double bond, respectively).
Bibliography

Abdelmagid, SA., SE. Clarke, DE. Nielsen, A. Badawi, A. El-Sohemy, DM. Mutch, and DWL. Ma. 2015. 'Comprehensive Profiling of Plasma Fatty Acid Concentrations in Young Healthy Canadian Adults', PLOS One, 10: e0128167.


Amin, AS., JR. Giudicessi, AJ. Tijsen, AM. Spanjaart, YJ. Beckman, CA. Klemens, MW. Tanck, JD. Kapplinger, N. Hofman, MF. Sinner, M. Muller, WJ. Wijnen, HL. Tan, CR. Bezzina, EE. Creemers, AAM. Wilde, MJ. Ackerman, and YM. Pinto. 2011. 'Variants in the 3' Untranslated Region of the KCNQ1-Encoded Kv7.1 Potassium Channel Modify Disease Severity in Patients with Type 1 Long QT Syndrome in an Allele-Specific Manner', European Heart Journal, 33: 714-23.

Anderson, BM., and DWL. Ma. 2009. 'Are All w-3 Polyunsaturated Fatty Acids Created Equal?', Lipids in Health and Disease, 8.


Bohannon, BM., ME. Perez, SI. Lin, and HP. Larsson. 2018. 'w-6 and w-9 Polyunsaturated Fatty Acids with Double Bonds Near the Carboxyl Head Have the Highest Affinity and Largest Effects on the Cardiac I\textsubscript{ks} Potassium Channel', Acta Physiologica.


Clinic, Cleveland. 2017. 'Sudden Cardiac Death (Sudden Cardiac Arrest)'.


Dick, IE., R. Joshi-Mukherjee, W. Yang, and DT. Yue. 2016. 'Arrhythmogenesis in Timothy Syndrome is Associated with Defects in Ca\textsuperscript{2+}-Dependent Inactivation', *Nature Communications*, 7.


Hofmann, F., V. Flockerzi, S. Kahl, and JW. Wegener. 2014. 'L-type Ca_{1.2} Calcium Channels: From In Vitro Findings to In Vivo Function', Physiology Reviews, 94: 23.

Horrocks, LA., and YK. Yeo. 1999. 'Health Benefits of Docosahexaenoic Acid (DHA)', Pharmacological Research, 40: 15.


Ko, SH., PW. Lenkowski, HC. Lee, JP. Mounsey, and MK. Patel. 2005. 'Modulation of Nav1.5 by Beta-1 and Beta-3 Subunit Co-Expression in Mammalian Cells', Pflugers, 449: 403-12.

Larsson, JE., HP. Larsson, and SI. Liin. 2018. 'KCNE1 Tunes the Sensitivity of Kv7.1 to Polyunsaturated Fatty Acids by Moving Turret Residues Close to the Binding Site', eLIFE.


Liin, SI., MS. Ejneby, R. Barro-Soria, Skarsfeldt MA., JE. Larsson, FS. Harlin, T. Parkkari, BH. Bentzen, N. Schmitt, HP. Larsson, and F. Elinder. 2015. 'Polyunsaturated Fatty Acid Analogs Act Antiarrhythmically on the Cardiac \( I_{\text{Ks}} \) Channel', *Proceedings of the National Academy of Sciences*, 112: 6.

Liin, SI., JE. Larsson, R. Barro-Soria, BH. Bentzen, and HP. Larsson. 2016. 'Fatty Acid Analogue N-arachidonoyl Taurine Restores Function of \( I_{\text{Ks}} \) Channels with Diverse Long QT Mutations', *eLIFE*.


Nguyen, PT., KR. DeMarco, I. Vorobyov, CE. Clancy, and V. Yarov-Yarovoy. 2019. 'Structural Basis for Antiarrhythmic Drug Interactions with the Human Cardiac Sodium Channel', *Proceedings of the National Academy of Sciences*, 116: 2945-54.


Pantazis, A., N. Savalli, D. Sigg, A. Neely, and R. Olcese. 2014. 'Functional Heterogeneity of the Four Voltage Sensors of a Human L-type Calcium Channel', *Proceedings of the National Academy of Sciences*, 111: 18381-86.
Pepe, S., K. Bogdanov, H. Hallaq, H. Spurgeon, A. Leaf, and E. Lakatta. 1994. 'w-3 Polyunsaturated Fatty Acid Modulates Dihydropyridine Effects on L-type Ca²⁺ Channels, Cytosolic Ca²⁺, and Contraction in Adult Rat Cardiac Myocytes', *Proceedings of the National Academy of Sciences*, 91: 8832-36.


Schwartz, PJ., MJ. Ackerman, and AAM. Wilde. 2017. 'Channelopathies as Causes of Sudden Cardiac Death', *Cardiac Electrophysiology Clinics*, 9: 537-49.


Tsukamoto, I., and S. Sugawara. 2018. 'Low Levels of Linoleic Acid and a-Linoleic Acid and High Levels of Arachidonic Acid in Plasma Phospholipids are Associated with Hypertension', *Biomedical Reports*, 8: 69-76.


Wessa, P. 2017. 'Hierarchical Clustering(v1.0.5) in Free Statistics Software(v1.2.1)'.


Xiao, YF., SN. Wright, GK. Wang, JP. Morgan, and A. Leaf. 2000. 'Coexpression with Beta-1-Subunit Modifies the Kinetics and Fatty Acid Block of hH1a Na\textsuperscript{+} Channels', American Journal of Physiology, 279: 12.


