Molecular mechanisms of modulation of K_v7 channels by polyunsaturated fatty acids and their analogues

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Cover picture shows linoleic acid (blue/purple), a polyunsaturated fatty acid, bound to Kv7.1 (grey) inserted in a membrane (green). Picture was created by Samira Yazdi.

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I. List of Papers

This thesis is based on the following papers:

- I. Liin SI, **Larsson JE**, Barro-Soria R, Bentzen BH and Larsson HP (2016) N-arachidonoyl taurine rescues diverse long QT syndrome associated I_{Ks} channel mutants. *Elife*. 2016 Sep 30;5. pii: e20272.
- II. **Larsson JE**, Larsson HP, Liin SI (2018) KCNE1 tunes the sensitivity of $K_v7.1$ to polyunsaturated fatty acids by moving turret residues close to the binding site. *Elife*. 2018 Jul 17;7. pii: e37257.
- III. **Larsson JE**, Karlsson U, Wu X, Liin SI (2020) Combining endocannabinoids with retigabine for enhanced effect on the M-channel and improved K_v7 subtype selectivity. *J Gen Physiol* 152 (8): e202012576.

II. Populärvetenskaplig sammanfattning

Elektrisk signalering är viktig för avancerade flercelliga organismer. Den möjliggör snabb signalering över långa avstånd som i våra nerver, koordinering av hjärtats muskelceller och bearbetning av information i vårt centrala nervsystem. Signaleringen sker genom elektrisk aktivering av celler genom att ändra spänningen mellan insidan och utsidan av cellen. Spänningen över cellens membran styrs genom att kontrollera nivån av positiva mot negativa laddningar i cellen. Detta kontrolleras via ändring av genomsläppligheten för positivt och negativt laddade joner genom membranet.

Genomsläppligheten för joner över cellmembranet regleras av membranbundna proteiner. Dessa proteiner kan öppnas och stängas för att ändra genomsläppligheten för olika joner utifrån olika signaler. En typ av proteiner som styr genomsläppligheten för joner kallas spänningskänsliga jonkanaler. En spänningskänslig jonkanal regleras av förändringar i spänningen över cellmembranet. Vanligtvis är spänningskänsliga jonkanaler stängda när cellen är i vila och öppnas av elektrisk aktivering.

Elektriskt retbara celler har i sitt vilotillstånd en negativ spänning över cellmembranet, dvs en övervikt av negativt laddade partiklar på insidan av cellen jämfört med cellens utsida. De två viktigaste jonerna för att reglera cellens elektriska aktivitet är natrium och kalium. Dessa har olika egenskaper: positivt laddade natriumjoner vill in i cellen för att öka mängden positiva laddningar för att elektriskt aktivera cellen, tvärtom vill positivt laddade kaliumjoner lämna cellen för att föra cellen tillbaka mot viloläget. Som ett exempel på dessa egenskaper sker följande om man stimulerar en nervcell elektriskt: först kommer spänningskänsliga natriumjonkanaler aktiveras och natriumjoner strömma in för att elektriskt aktivera cellen. Efter viss fördröjning öppnar även spänningskänsliga kaliumjonkanaler, kaliumjoner lämnar cellen vilket för spänningen tillbaka till vilopotential och avslutar den elektriska aktiviteten. Båda dessa kanaltyper är därför viktiga för att kontrollera den elektriska aktiviteten i nervceller.

Denna avhandling studerar spänningskänsliga kaliumjonkanaler. Spänningskänsliga kaliumjonkanaler är en stor familj av olika proteiner som är besläktade. Dessa är spridda i olika vävnader i kroppen och uppfyller olika funktioner. Avhandlingen fokuserar på den sjunde familjen av spänningskänsliga kaliumjonkanaler (Kv7). Kv7-familjen har 5 olika närbesläktade proteiner, Kv7.1 till Kv7.5. Dessa uppfyller olika funktioner, till exempel är Kv7.1 viktig för avslutandet av den elektriska aktiviteten i hjärtat medan Kv7.2 och Kv7.3 är viktiga för retbarheten i centrala nervsystemet. Kv7-kanalerna kopplas till flera former av sjukdom bland annat via mutationer i generna som kodar för kanalerna. Denna sjukdomskoppling gör Kv7-kanalerna intressanta att studera, dels för att studera vad som händer i kanalen vid mutationer som orsakar sjukdom men även som måltavla i utveckling av ämnen som kan användas som framtida läkemedel.

Vi studerar spänningskänsliga kaliumjonkanaler i ett cellsystem baserat på oocyter (groddagg) från den afrikanska klogrodan *Xenopus laevis*. Vi använder dessa groddagg för att bygga miljontals kopior av mänskliga jonkanaler och placera dem i oocyters cellmembran. Kanalerna studeras sedan via en metod som heter två elektrods voltage clamp. Via denna metod kontrolleras spänningen över cellmembranet hos groddägg för att öppna och stänga kanalerna samt för att mäta jonströmmen som produceras av kanalerna vid olika spänningar. Vi jämför hur olika ämnen påverkar jonströmmen genom kanalerna, detta nyttjas av oss för att hitta ämnen som aktiverar jonkanalerna.

Vi är intresserade av att hitta ämnen som påverkar jonkanaler och som i framtiden potentiellt kan användas som läkemedel mot sjukdomar orsakade av dysfunktionella jonkanaler. Från tidigare studier vet vi att fleromättade fettsyror (PUFA) aktiverar spänningsskänsliga kaliumjonkanaler. PUFA finns bland annat i fiskleverolja från exempelvis lax. Intag av dessa fettsyror i kosten har länge visat sig ha en skyddande effekt mot hjärtsjukdomar. Det är dock flera aspekter av hur PUFA utövar sin effekt som är oklara.

Vi vet sedan tidigare att PUFA aktiverar Kv7-kanaler. PUFA aktiverar Kv7-kanaler genom att påverka kanalens reaktion på spänningsförändringar samt öka kanalernas genomsläpplighet för kaliumjoner. Fettsyrorna kan till exempel göra att kanalerna öppnas vid mer negativa spänningar. Det finns dock fortfarande frågetecken kring mekanismen för aktivering av Kv7-kanaler med PUFA. Mitt mål med denna avhandling är att studera detaljer i hur PUFA utövar sin aktiverande effekt på Kv7-kanaler. I synnerhet vill vi veta mer om fettsyrornas mekanism för aktivering av kanalerna samt hur proteiner som reglerar Kv7-kanaler påverkar effekten. Vi vill även utreda om dessa ämnen utgör lämpliga kandidater som framtida läkemedel.

Avhandlingens första mål är att studera och identifiera delar av kanalen som är viktiga för samspelet mellan kanalen och fettsyran. Vi visar varför det reglerade KCNE1-proteinet tar bort effekten av PUFA på Kv7.1. Detta sker via att KCNE1 ändrar den lokala miljön kring fettsyran så fettsyran förlorar sin negativa laddning, den negativa laddningen är viktig för att PUFA ska kunna verka på Kv7-kanaler. KCNE1 inducerar en förändring av kanalens form som leder till att en del av kanalen kommer närmare bindningsplatsen för fettsyran, denna del av kanalen är viktig för att ändra den lokala miljön.

Vi visar även att den aktiverande mekanismen för PUFA och PUFA-besläktade ämnen som beskrivits på Kv7.1 också stämmer för Kv7.2 och till viss del på Kv7.3. Vi visar att positivt laddade aminosyror på kanalens utsida är viktiga för effekten av fettsyror, borttagning av dessa aminosyror sänker den aktiverade effekten.

I avhandlingen studerar vi även vilken påverkan olika sjukdomsorsakande mutationer har på kanalerna samt försöker återställa kanalernas funktion. Vi visar att mutationer i Kv7.1 skapar dysfunktion via att påverka kanalens reaktion på spänningsförändringar och ändra hur snabbt kanalen öppnar och stänger. Olika mutationer påverkar kanalen på olika sätt. Vissa mutationer påverkar kanalen genom att påverka kanalens möjlighet att uppfatta spänningsförändringar och andra via att påverka kanalens möjlighet att koppla spänningsförändringar till öppning av kanalen. Den PUFA-besläktade substansen N-AT aktiverar Kv7-kanaler med sjukdomsorsakande mutationer. N-ATs aktivering leder till att de muterade kanalerna återfår en funktion som liknar den för den friska kanalen.

Till sist undersöker vi om kombinationer av olika kaliumjonkanalsaktiverare kan nyttjas för att styra effekten till endast utvalda Kv7-kanaler. Till denna studie använder vi ett potent ämne som är besläktat med PUFA, ARA-S. Vi visar att när ARA-S kombineras med andra kända Kv7-aktiverare kan vi behålla aktiveringen av Kv7-kanaler som normalt återfinns i nervsystemet och samtidigt minska aktivering av Kv7-kanaler som normalt återfinns i hjärta och i urinblåsan. Denna kombinationsstrategi skulle kunna nyttjas för att behandla epilepsi som kopplas till de neuronala kanalerna utan att riskera bieffekter från aktivering av övriga kanaler.

Sammanfattningsvis, PUFA och PUFA-besläktade ämnen aktiverar Kv7-kanaler. PUFA och PUFA-besläktade ämnens aktiverade effekt kan bidra till utvecklingen av framtida läkemedel mot exempelvis arytmier i hjärta eller epilepsi.

III. Abstract

Ion channels are membrane proteins that regulate the permeability of ions across the cell membrane. The sequential opening of different types of ion channels produces action potentials in excitable cells. Action potentials are a way for the body to, for example, transmit signals quickly over a long distance.

The Kv7 family is an important group of voltage-gated potassium channels. Mutations that cause dysfunction in members of the Kv7 family are associated with several forms of disease. Compounds that can activate Kv7 channels have previously been shown to work as medical treatments. However, the previously available antiepileptic drug retigabine, has been withdrawn due to adverse effects. Thus, there is a need for further development of compounds that target these channels. PUFA and PUFA analogs have previously been demonstrated to activate Kv7.1 through an electrostatic mechanism. This thesis investigates new aspects of the interaction between Kv7 channels and PUFA-related compounds.

The data in this thesis are from human Kv7 channels expressed in *Xenopus laevis* oocytes. The currents produced by the channels expressed in the oocytes have been studied using two-electrode voltage clamp. Our aim was to study the mechanism for the activation of Kv7 channels by PUFA and PUFA analogs. More specifically, we intended to study why the beta subunit KCNE1 abolishes the activating effect of PUFA on Kv7.1 and how PUFAs activate Kv7.2 and Kv7.3. Additionally, we wanted to study aspects that may affect whether these compounds are viable as medical treatments. For instance, whether these compounds can activate channels containing disease-causing mutations and whether we can improve compound selectivity towards certain Kv7 channels.

In Paper I, we introduce disease-causing mutations found in patients into Kv7.1 and KCNE1. The characterization showed that these channels had altered biophysical properties compared to wild type channels. A PUFA analog was found to activate and, to a large degree, restore wild type-like biophysical properties in the mutated channels regardless of the localization of the mutation in the channel.

In Paper II, we demonstrate why PUFA is unable to activate Kv7.1 co-expressed with beta subunit KCNE1. KCNE1 induces a conformational change of Kv7.1 that moves the S5-P-helix loop closer to the PUFA binding site. This causes negative charges of the loop to attract protons that reduce local pH at the PUFA binding site. The decreased local pH leads to protonation of PUFA and the PUFAs therefore lose their negative charge. Thus, PUFA cannot activate Kv7.1 when it is co-expressed with KCNE1.

In Paper III, we study a group of PUFA-related substances, endocannabinoids, on Kv7 channels. One endocannabinoid, Arachidonoyl-L-Serine (ARA-S), was identified as a potent activator of the neuronal M-channel, comprising Kv7.2 and Kv7.3 heteromers. We study the activating mechanism of ARA-S in Kv7.2 and Kv7.3, demonstrating how the activating effect is linked to two parts of the channel protein, one in the voltage sensor domain and the other in the pore domain. ARA-S was also found to activate Kv7.1 and Kv7.5 but not Kv7.4, which instead was inhibited. Retigabine, a compound that activates the M-channel but has a different Kv7 subtype selectivity compared to ARA-S, was used in combination with ARA-S to maintain a potent effect on the M-channel while limiting the activation of other Kv7 channels.

In conclusion, the activating effect of PUFA analogs on Kv7 channels may be helpful in the development of future drug candidates for diseases such as arrhythmia and epilepsy.

1. Introduction

Electrical signals are vital for rapid long-distance signaling in peripheral nerves and coordination of cells in the heart. One of the first findings to describe the influence of electricity in living things was in 1791, when Luigi Galvani demonstrated that electrical stimulation could trigger muscle movement in a dead frog (Galvani 1791). Another early discovery of the importance of electricity was made by Emil Du Bois-Reymond. He discovered that the passage of signals in nerves is accompanied by an electrical discharge, currently known as the action potential (Du Bois-Reymond 1849). The first clue that ions were involved in electrical signaling was presented by Sydney Ringer. In his experiments on isolated hearts, he showed that sodium, potassium and calcium ions are all important to maintaining the heartbeat (Ringer 1882). Julius Bernstein proposed that the resting potential and action potential in nerves can be explained by changes in permeability to ions over the cell membrane (Bernstein 1902). This work was further developed by Hodgkin and Katz showing that an increased permeability to sodium ions could evoke an action potential (Hodgkin and Katz 1949). During the early 1950s, Hodgkin and Huxley presented their work on the giant squid axon. They found that sodium, potassium and leak currents together can form an action potential (Hodgkin and Huxley 1952a, Hodgkin and Huxley 1952b, Hodgkin and Huxley 1952c, Hodgkin and Huxley 1952d): This finding was awarded a Nobel prize. It has since been shown that the permeability of ions is regulated by ion channels (Narahashi et al. 1964, Armstrong and Binstock 1965). Today, these channels are cloned and expressed, enabling studies of the channels using electrophysiological techniques (Noda et al. 1984, Noda et al. 1986, Tanabe et al. 1987, Tempel et al. 1987). Mechanisms for the opening and closing of channels have been proposed and the number of solved structures for ion channels is increasing every year. The field of electrophysiology has come far, although there is still scope for further exploration.

This thesis concerns voltage-gated potassium (Kv) channels and how to regulate the activity of such channels using compounds. The activity of Kv channels are vital for the normal activity of the nervous system and the heart. Thus, it is common for mutations in the genes coding for Kv channels to cause inherited diseases, for example, epilepsy and arrhythmias. A possible way to treat these diseases would be to use drugs that activate Kv channels. However, at the time this thesis is being written, there are no widely used drugs that activate Kv channels. My work concerns how polyunsaturated fatty acids (PUFA) and PUFA analogs activate Kv channels, focusing on the Kv7 family. The introduction to the thesis will summarize the current knowledge on Kv channels including structure, voltage gating and the regulation of Kv channels with small molecule compounds. Each section will start with Kv channels in general followed by details of the Kv7 family.

1.1 Physiological role of Kv channels

The fatty nature of the membrane prevents the passage of charged ions across the cell membrane. Thus, there is a need for ion channels for the transmembrane passage of ions. There are several types of potassium channels that are controlled by, for example, calcium or ATP. However, this thesis will focus on Kv channels. Kv channels make up a large and diverse group in comparison to voltage-gated sodium (Nav) channels and voltage-gated calcium (Cav) channels. Kv channels are expressed in all kinds of tissues in the body, with varying functions. There is a total of 12 Kv families, called Kv1-12 (Hille 2001).

The Kv7 family is encoded by the KCNQ genes. The family contains five isoforms. Kv7.1, Kv7.2, Kv7.3, Kv7.4 and Kv7.5 are coded by corresponding KCNQ genes (KCNQ1-5) (Robbins 2001).

Kv7.1 is expressed in various parts of the body but is primarily known to be important in the heart and the inner ear (Barrese et al. 2018). Kv7.1, as part of the channel generating the I_{Ks} current, provides a delayed repolarizing current in the heart (Barhanin et al. 1996, Sanguinetti et al. 1996) and maintains ionic balance in the inner ear (Neyroud et al. 1997). It is also expressed in the kidney, intestine, pancreas and lung (Barrese et al. 2018). The different roles of Kv7.1 are enabled by interactions with KCNE subunits.

Kv7.2 and Kv7.3 are both neuronal channels (Wang et al. 1998, Cooper et al. 2000). Kv7.2 can form functional channels by itself but is normally found in heterotetramers with Kv7.3 (Wang et al. 1998). Kv7.3 can form homotetramers but produces a very weak current by itself (Wang et al. 1998). Kv7.2 and Kv7.3 as heterotetramers contribute to a neuronal current called the M-current (Wang et al. 1998, Cooper et al. 2000). The M-current stabilizes the subthreshold membrane potential of neurons, limiting the neuron's ability to fire action potentials.

Kv7.4 is best known for maintaining a proper potassium level in the inner ear (Kubisch et al. 1999, Kharkovets et al. 2000). It is also expressed to a lesser extent in the heart and other muscle tissue, for example, the smooth musculature of the bladder (Barrese et al. 2018).

Kv7.5 is found in both nervous and muscle tissue (Lerche et al. 2000, Chadha et al. 2014). In nervous tissue it contributes to the M-current as heterotetramers with Kv7.3. In certain tissues, Kv7.4 and Kv7.5 can form heterotetramers, for example, to regulate smooth muscle contraction (Provence et al. 2018).

Kv7 channels can be regulated by forming complexes with so-called beta subunits; the most known beta subunit for Kv7 channels are called KCNE proteins. KCNE proteins are single transmembrane proteins that were initially thought to produce potassium channels by themselves (Takumi et al. 1988). Today, it has been shown that, rather than forming channels by themselves, KCNE proteins regulate the function of other channels as subunits in a complex (Abbott 2016). There are five known KCNE proteins, KCNE1-5. Not all Kv channels are known to be modulated by KCNE proteins, but several can be regulated by multiple KCNE types. Best known is the interactions with Kv7.1, which is differently regulated by each KCNE isoform. Typical changes involve altered voltage dependence and altered kinetics of channel opening (Abbott 2016).

1.2 Structure of Kv channels

The general structure of a voltage-gated ion channel is a central pore with four surrounding voltage-sensing domains (VSD) (Figure 1A) (Long et al. 2005). For Kv channels the most common structure is comprised of four separate but identical proteins together forming a functional channel in which each of the proteins contributing to the main channel structure is called an alpha subunit. Each alpha subunit contains six transmembrane segments referred to as S1–S6 (Figure 1B). Between segments 5 and 6 is a single pore loop. Before and after the transmembrane segments are the N-terminal and C-terminal domains of varying length.

For Kv channels, transmembrane segments 1 to 4 (S1–S4) form the VSD while transmembrane segment 5 (S5), the pore loop, and transmembrane segment 6 (S6) of each subunit together form the pore domain (PD). S4 of the VSD contains four to seven positively charged residues, most commonly arginines. The positive charges are usually located at three-residue intervals along the length of S4. The PD forms a water-filled pore that extends across

the membrane and connects the extracellular solution to the intracellular solution. The PD also contains the gate that is usually located at the bottom of S6; the gate controls the opening and closing of the pore (Doyle et al. 1998). The PD also contains a selectivity filter that limits the conductance to certain ion species (Hille 1972). Since the mid-2000s, the overall structure of K_v channels has been known due to a series of X-ray crystallography studies of potassium channels (Long et al. 2005, Long et al. 2007).

The structure of K_v7 channels follows the general structure of K_v channels. KCNQ genes code for proteins with six transmembrane segments. These proteins form functional channels as tetramers. The S4 of K_v7 channels has four to six positively charged residues. K_v7.1 has four while K_v7.2-5 has six positive charges (Robbins 2001). In 2017 the structure of K_v7.1 was solved (Sun and MacKinnon 2017). The available structure reveals a domain-swapped channel, meaning that the VSD of a subunit is adjacent to segments of the PD from another subunit (Figure 1C).

Figure 1. (A) Top view cartoon of the general structure of a voltage sensitive ion channel. Four voltage-sensing domains (green) surround a central pore (blue). (B) Cartoon of the transmembrane segments of a voltage-gated potassium channel. Segments 1–4 (S1–S4) form the VSD while segments 5–6 contribute to the central PD. Also, cartoon of the single-transmembrane segment of KCNE1. (C) Structure of K_v7.1 from Sun and MacKinnon 2017 (PDB 5VMA). Each subunit is colored differently (red, blue, yellow and green). Note the domain-swapped structure in which the voltage sensor of a subunit is close to the neighboring pore domain. (D) Structure of K_v7.1 in complex with KCNE3 from Sun and MacKinnon 2020 (PDB 6V00). K_v7.1 VSD in green, K_v7.1 pore domain in blue and KCNE3 in orange. The C-termini of the channel and KCNE3 have been removed from the figure. (E) Side view of K_v7.1 in complex with KCNE3; same coloring as in D. The C-termini of the channel and KCNE3 have been removed from the figure.

Figure 1B shows a schematic version of the single-transmembrane structure of KCNE subunits. The structure of K_v7.1 with KCNE subunits has been solved for K_v7.1 with KCNE3. KCNE3 binds in a pocket formed by S1, S5 and S6 of three different subunits (Figure 1D&E) (Sun and MacKinnon 2020). KCNE1, an auxiliary subunit to K_v7.1 in the heart and ear, is thought to bind to the channel in a similar location as KCNE3. This assumption is based on proposed models from molecular-dynamics simulations of K_v7.1

together with KCNE1 (Xu et al. 2013) and experimental data (Chung et al. 2009, Xu et al. 2013). The exact number of KCNE subunits per channel has been debated, but has been suggested to be between 2–4 KCNE1 subunits per Kv7.1 tetramer (Nakajo et al. 2010, Plant et al. 2014, Murray et al. 2016). In the structure of Kv7.1 with KCNE3, four KCNE3 subunits were bound to the channel (Sun and MacKinnon 2020).

1.3 Voltage gating of Kv channels

Kv channels are voltage gated. This means that they are gated through change of the voltage across the cell membrane. The change of voltage triggers a conformational change of the VSD; the conformational change is coupled to the opening of the pore. In a simplified model, a Kv channel can be in four different states (Figure 2):

1. At resting potential the VSD is in a resting position, and the pore is closed (resting state).
2. Depolarization induces a conformational change of the VSD into an activated conformation, the pore is closed (activated state).
3. The conformational change of the VSD triggers an opening of the pore (open state).
4. Some Kv channels inactivate after opening, shutting down conductance. The VSD is still in the activated conformation while the channel is inactivated (inactivated state).

When the membrane potential returns to its resting potential, the channel returns to the resting state. The following section will cover important structures for voltage gating and the different steps of voltage gating leading up to the opening (and in some cases inactivation) of the pore.

Figure 2. Schematic cartoon of the four states of a Kv channel. Depolarization causes movement of the positive charges in the voltage sensor leading to activation and subsequent pore opening (and in some cases inactivation). Repolarization returns the voltage sensor to its resting state.

1.3.1 Voltage sensor activation

Membrane depolarization triggers outward S4 movement. Hodgkin and Huxley predicted that the activation of ion channels was due to the movement of charged particles (Hodgkin and Huxley 1952b). However, among the first evidence of this prediction was the recording of gating currents made in the early 1970s (Armstrong and Bezanilla 1973, Keynes and Rojas 1974). Gating currents are small transient currents generated by the movement of the charges in S4 that can be recorded immediately after depolarization.

When the membrane is depolarized, S4 moves through the membrane, following the electrostatic force, from its resting downward position to an activated outward position

(Figure 2). For many years it was debated how this conformational change of the VSD occurs. Currently, the consensus model for the movement of S4 is called the sliding helix model (Catterall 1986, Guy and Seetharamulu 1986, Vargas et al. 2012, Wisedchaisri et al. 2019). According to this model, S4 rotates and moves outwards during depolarization. The positive charges in S4 serve as gating charges that drive the movement. The movement occurs stepwise, with positive charges forming salt bridges with different negative counter charges to create a low energy pathway through the membrane (Papazian et al. 1995, DeCaen et al. 2008, DeCaen et al. 2009, Henrion et al. 2012). Different pairs form salt bridges in each step of the movement. S4 in the Shaker Kv channel is suggested to move approximately 12 Ångström from its resting to its activated position (Henrion et al. 2012).

In addition to the general principles described above, it has been described that the S4 movement of Kv7.1 is split into two separate parts occurring at different voltage ranges (Zaydman et al. 2014). It has been suggested that these movements correspond to the transitions between three distinct conformations of S4: resting, intermediate and activated. These three conformations have been described experimentally by showing that the S2 countercharge E160 interacts with a different positive charge in S4 in each conformation. The distribution of the positive charges in S4 of the Kv7 channels can be seen in figure 3A. In the resting state, E160 interacts with R228 (R1). In the intermediate state, E160 interacts with R231 (R2). In the activated state, E160 interacts with R237 (R4) (Figure 3B) (Zaydman et al. 2014). The activated state had been caught in cryo-EM structures (Sun and MacKinnon 2017, Sun and MacKinnon 2020) and the intermediate state has been shown in NMR spectroscopy (Taylor et al. 2020). Other interactions between S4 arginines and negative counter charges for the three states have also been suggested (Taylor et al. 2020). The S4 movements of Kv7.1 changes, depending on whether or not KCNE subunits are present. KCNE1 separates the two S4 movements, further clarifying the difference between the voltage ranges in which each S4 movement occurs (Barro-Soria et al. 2014, Taylor et al. 2020). KCNE3 shifts the first S4 movement to very negative voltages (Barro-Soria et al. 2015, Taylor et al. 2020).

Figure 3. (A) Sequence alignment for S4 of each Kv7 family member. The shaded area of the sequence corresponds to the helical S4. Gating charges are indicated in blue. A difference between Kv7 channels and other voltage-gated channels is that in the third natural position for an arginine counting from the top of S4, there is a neutral glutamine (Q3) in the Kv7 channels. (B) Shows the S4 helix of Kv7.1 in which the positive charges have been marked in blue. According to the sliding helix model, S4 moves outwards and also rotates during depolarization. Salt bridges are formed between the arginines in S4 and negative counter charges in S1–S3: different salt bridges are formed in each state during the outward movement. The figure shows the three states of the voltage sensor in Kv7.1: resting, intermediate and activated. Different arginines form salt bridges to the S2 countercharge E160 in each state.

1.3.2 Coupling the voltage sensor movement to the pore opening

There are several potential ways of coupling the movement of the S4 to the opening of the pore. Traditionally, the most important coupling is thought to go through the S4–S5 linker, which is called the canonical coupling mechanism. The intracellular S4–S5 linker is in direct contact with S6 of the PD of the same subunit. Studies suggest that a FYF motif in S6 forms a hydrophobic pocket into which the S4–S5 linker inserts (Labro et al. 2008, Haddad and Blunck 2011). This interaction couples the movement of S4 to a movement of the S4–S5 linker and this movement bends the gate open (Lu et al. 2002). However, the exact mechanism is not entirely known.

An alternative coupling mechanism proposes that the movement of S4 is coupled through neighboring transmembrane segments, sometimes referred to as non-canonical coupling. For domain swapped channels, such as the Kv7 channels, this would mean that the VSD from one subunit interacts with segments of the PD from a neighboring subunit. A possible coupling mechanism could involve the rotation of S4, causing displacement of the neighboring S5 segment, or that the upwards movement of the S4 changes the overall structure of the ion channel. These changes in PD conformations could cause the gate to open. There are studies that support this mechanism for certain ion channels (Soler-Llavina et al. 2006, Fernandez-Marino et al. 2018, Carvalho-de-Souza and Bezánilla 2019).

Both canonical and non-canonical coupling mechanisms could contribute to the opening of a voltage-gated channel. For Kv7.1, it has recently been shown that the coupling goes through both a canonical coupling and a newly described non-canonical coupling. The junction at which S4 and the S4–S5 linker meet is important for the non-canonical coupling in Kv7.1. This junction interacts with transmembrane parts of S5 and S6 in a neighboring subunit (Hou et al. 2020). For both coupling mechanisms to work it has been suggested that the lipid PIP₂ must be present in the inner bilayer of the cell membrane. Without the presence of PIP₂, S4 is still able to move but unable to couple its movement to the opening of the pore (Zaydman et al. 2013, Kasimova et al. 2015). All Kv7 channels require PIP₂ to function properly (Zhang et al. 2003, Kim et al. 2017).

1.3.3 Pore opening

The pore of Kv channels is open for the conduction of ions when the gate is in its open conformation and neither of the inactivation mechanisms have engaged (see 1.3.4 for inactivation). The gate of the pore comprises a bundle crossing formed by the lower parts of all four S6 helices, sometimes described as an upside-down teepee-like arrangement (Doyle et al. 1998). The bundle crossing limits the pore diameter to prevent conduction. The movement of S4 leads to widening of the bundle crossing and this widening permits ion conduction. This gate structure has been seen in crystal structures of ion channels in the superfamily of voltage-gated ion channels (Long et al. 2005, Wisedchaisri et al. 2019).

Kv7 channels have a bundle crossing of S6 in the pore that functions as a gate (Sun and MacKinnon 2017). Kv7.1 can open its gate when S4 is in its intermediate and activated state. The two open conformations are usually referred to as intermediate open (IO) and activated open (AO) (Zaydman et al. 2014). In Kv7.1, IO is the dominant open conformation. The two open states are coupled differently: IO is dependent on canonical coupling while AO is dependent on both the canonical and the non-canonical coupling (Hou et al. 2020). An alternative proposed mechanism is that Kv7.1 can open before all VSDs have reached the activated position (Osteen et al. 2012). When Kv7.1 is co-expressed with KCNE1 (Kv7.1 + E1), channel opening is limited to AO (Zaydman et al. 2014). KCNE1 also changes the properties of the AO state.

1.3.4 Inactivation mechanisms

The inactivated state is a non-conducting state that differs from the closed state. In the inactivated state, the VSD is in the activated conformation and a different mechanism causes the pore to stop conducting. When the channel enters the inactivated state, it can cause the channel to be unable to re-open until it has been returned to the resting state.

Inactivation is separated into two main types: fast (N-type) or slow (C-type) inactivation. The fast inactivation stops conduction by a ball-and-chain mechanism (Armstrong and Bezanilla 1977). A cluster of amino acids (ball) is attached to the main protein by a string of residues (chain) on the intracellular side. The ball can enter the intracellular side of the pore once it is open, bind to the bottom part of the vestibule and create a physical plug. The ball must release from the pore in order for the channel to re-open. This mechanism is called N-type inactivation due to structures in the N-terminus of the Shaker Kv channel being linked to the inactivation and removing said parts of the N-terminus can abolish fast inactivation (Hoshi et al. 1990).

Slow (C-type) inactivation occurs at the selectivity filter in the extracellular part of the pore. The mechanism for this inactivation is thought to be a rearrangement of the selectivity filter that prevents the flow of ions (Starkus et al. 1997, Li et al. 2018). The exact mechanism for slow inactivation is still subject to debate. It was originally thought that parts of the C-terminus were responsible, which is why it is called C-type.

Inactivation has been reported for Kv7.1 but does not exhibit the classical hallmarks of either N-type or C-type inactivation (Meisel et al. 2018). It has been proposed that the inactivation mechanism for Kv7.1 stems from the transition from the intermediate open state (IO) to the activated open state (AO) (Hou et al. 2017). This transition is thought to decrease the open probability of the channel without changing the single channel conductance. AO occurs at higher voltages. Thus, inactivation becomes exaggerated upon longer pulsing to higher voltages. KCNE1 co-expression suppresses IO in Kv7.1 + E1, only permitting the AO state. Thus, inactivation is not seen in Kv7.1 + E1, since the transition between IO and AO does not occur. Kv7.2 and Kv7.3 have not been shown to inactivate whereas Kv7.4 and Kv7.5 have been reported to inactivate to a small degree (Jensen et al. 2007).

1.4 Kv channels in disease

There are many diseases associated with Kv channels, both inherited diseases caused by mutations in genes for Kv7 channels as well as problems caused by compounds targeting channels. Mutations in the genes coding for Kv channels have been associated with diseases such as epilepsy and arrhythmias (Hedley et al. 2009, Chen et al. 2017). The mutations can cause problems by changing expression rate, transportation of the channel to the cell membrane or altering the function of the channel. Both increased and decreased activity of the channels can cause disease (Hedley et al. 2009). Kv channels can also be targeted by various compounds found in nature or made by man. Venomous animals produce toxins that can bind to and disrupt the function of channels (Luo et al. 2018). Adverse effects of drugs binding to Kv channels as a non-desired secondary target are not uncommon. For example, Kv11.1 (the hERG channel) is a cardiac channel known to be blocked by compounds with different structures, which is a common cause of arrhythmias (Stansfeld et al. 2006).

There are several forms of disease associated with mutations in the KCNQ genes. These diseases originate in different tissues. A selection of tissues expressing Kv7 channels have been summarized in figure 4. Below is a brief summary of the diseases that have been associated with the different KCNQ genes.

Mutations in KCNQ1 have been associated with cardiac arrhythmias such as long QT syndrome (LQTS), short QT syndrome and familial atrial fibrillation (Tsai et al. 2008, Hedley et al. 2009). They have also been associated with a syndrome called Jervell and Lange-Nielsen syndrome, consisting of LQTS in combination with sensorineural hearing loss (Nakano and Shimizu 2016). Long/short QT syndrome are arrhythmias caused by delayed/accelerated repolarization of the heart ventricle, respectively, visualized as a change in QT time on an ECG. The change in QT time in the case of KCNQ1 mutations is due to the altered activity of the late repolarizing current, I_{Ks} ($K_v7.1 + E1$).

KCNQ2/ $K_v7.2$ and KCNQ3/ $K_v7.3$ are associated with benign familial neonatal convulsions (BFNC) and epilepsy (Chen et al. 2017). BFNC is a form of epilepsy that manifests as tonic-clonic seizures during the first months of life and with increased risk of seizures later in life. Mutations in either gene can increase the excitability of the neuron, increasing the risk of epilepsy. This is because of the altered function of the M-current that is generated by heteromers of $K_v7.2$ and $K_v7.3$ ($K_v7.2/3$).

Mutations in the gene coding for $K_v7.4$ (KCNQ4) are associated with non-syndromic sensorineural deafness type 2, an inherited form of progressive hearing loss (Nie 2008). Dysfunction of $K_v7.5$ has been associated with mental retardation and epilepsy (Lehman et al. 2017).

Figure 4. Figure indicating the expression of K_v7 channels in certain tissues. It also indicates different forms of disease associated with the dysfunction of named channels. The figure has been adapted from a design by Freepik.

1.5 Modulation of K_v channels

Since many conditions such as arrhythmias, epilepsy and pain could be caused by the dysfunctional excitability of cells, for many years ion channels have been a primary target for drug development (Overington et al. 2006). Many drugs available on the market target ion channels, both ligand- and voltage-gated channels. Nav and Cav channel blockers have been

used in clinics for a long time (Catterall and Swanson 2015). More recently, K_v channel activators have been suggested as possible treatments.

The following section will summarize potential ways of modulating K_v and K_v7 channels using small molecule compounds. The section will start by presenting inhibiting compounds and then compounds that increase K_v7 channel activity.

1.5.1 Inhibitors

The classical inhibitor of ion channels is the pore blocker. Pore blockers prevent the passage of ions through the channel by blocking the pore. Possibly the most known are local anesthetics such as lidocaine, a blocker of Na_v channels. Lidocaine was developed in Sweden in the 1940s (Löfgren 1948). A number of antiarrhythmics block the K_v channels. For the K_v7 family, the two most known blockers are chromanol 293B (an I_{Ks} blocker) and XE991 (an M-channel blocker). Chromanol 293B is thought to bind to the selectivity filter (Lerche et al. 2007) while the XE991 binding site has yet to be determined.

1.5.2 K_v7 channel activators

Over the course of the last two decades the K_v channels, particularly the K_v7 channels, have been popular targets in drug development. K_v7 channel activators are thought to have potential antiarrhythmic, antiepileptic and pain-relieving effects (Wu and Sanguinetti 2016, Barrese et al. 2018). Currently, the most well-known compound is retigabine, which proved that K_v7 channels are a viable target for antiepileptic treatments. However, several other compounds have been demonstrated to activate K_v7 channels. There are activating compounds that target the VSD and/or the PD. This suggests that there are several different ways that a compound could activate a K_v7 channel. Below is a brief summary of some of the more known compounds that target K_v7 channels.

Retigabine was discovered in the 1990s as an anticonvulsant acting on the neuronal M-channel ($K_v7.2/3$) (Rostock et al. 1996, Rundfeldt 1997). It was approved as an antiepileptic treatment by the FDA in 2011 under the name Potiga for adjunctive treatment of partial-onset epileptic seizures. Due to adverse effects such as blue discoloration of mucus tissue, its use was limited to pharmacoresistant epilepsy and in 2017 retigabine was withdrawn from the market. Since this time there have been ongoing investigations to identify suitable analogs of retigabine that reduce the risk of adverse effects. Retigabine is still used in academia as a tool to study K_v7 channels.

Retigabine activates the $K_v7.2/3$ channel by shifting the voltage dependence of the opening towards more negative potentials (Main et al. 2000). Apart from $K_v7.1$, all K_v7 channels can be activated by retigabine. $K_v7.3$ is the most sensitive to retigabine, followed by $K_v7.2$, $K_v7.4$ and $K_v7.5$ (Schenzer et al. 2005). Retigabine targets the pore of the channel. A tryptophan in S6 has been found to be important for the activation, W236 for $K_v7.2$ and W265 for $K_v7.3$ (Schenzer et al. 2005, Wuttke et al. 2005). A corresponding tryptophan residue is not found in $K_v7.1$, which is why it is insensitive to retigabine. Figure 5A shows this tryptophan residue along with additional residues that have been suggested to be important for the effect of retigabine in $K_v7.2$. The residues form a cluster in the pore in which it is suggested that retigabine binds.

Flupirtine is an analog of retigabine. It was introduced in Europe in 1984 as a non-opioid pain killer. Its main mechanism is potassium channel activation, mainly through the M-channel, but it has also been reported to modulate the $GABA_A$ receptor (Wickenden et al. 2004, Szelenyi 2013).

ICA-069673 (ICA73) is a pyrimidine benzamide analog that is a potent activator of Kv7.2/3 but is unable to activate Kv7.1 (Amato et al. 2011). ICA73 activates Kv7.2 strongly, while Kv7.3 shows only minor effects (Wang et al. 2017). ICA73 interacts with certain amino acids in S3 of the VSD of Kv7.2, namely, F168 and A181, see figure 5A (Wang et al. 2017). The effect of ICA73 is abolished or decreased in Kv7.2 if the aforementioned positions are mutated into the corresponding amino acids present in Kv7.3, F168L and A181P (Wang et al. 2017).

For Kv7.1, several small molecule activators have been proposed over the years, for example, R-L3, ML277, Zinc pyrithione (ZnPy), mefenamic acid and DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) (Busch et al. 1997, Salata et al. 1998, Gao et al. 2008, Yu et al. 2013). These compounds exhibit varying effects on Kv7.1, Kv7.1 + E1 and other Kv7 channels. It has been suggested that R-L3, ML277 and ZnPy bind to a pocket in the same area to which KCNE1 binds, see figure 5C (Seeböhm et al. 2003, Gao et al. 2008, Xu et al. 2015). Mefenamic acid and DIDS are thought to interact with residues 39–43 in the N-terminus of KCNE1 (Abitbol et al. 1999).

Figure 5. (A) Structure of Kv7.2 (Top and side view) with highlighted amino acids that are important for the effect of the Kv7.2 activators in B. Red residues are important for retigabine effects: W236, L275, L299, G301 (Schenzer et al. 2005, Wuttke et al. 2005, Lange et al. 2009), yellow residues for ICA73 effects: F168 and A181 (Wang et al. 2017). Residues important for retigabine are found in the pore domain while residues important for ICA73 are found in the voltage sensor domain (B) Molecular structure of the Kv7.2 activators retigabine and ICA73. (C) Structure of Kv7.1 (top and side view) with highlighted amino acids that are important for the effect of the Kv7.1 activators in D. Red residues are important for ML277 effects: F275, F332 and F335 (Xu et al. 2015), orange residues for R-L3 effects: G306 (Seeböhm et al. 2003), yellow residues for ZnPy effects: S338 and L342 (Gao et al. 2008). The highlighted residues are in a common binding area shared by all three compounds. (D) Molecular structure of the Kv7.1 activators ML277, R-L3 and ZnPy.

1.6 Polyunsaturated fatty acids (PUFA) and PUFA analogs

Even though there are several known antiepileptics and antiarrhythmics, there is still an obvious need for further development since a high proportion of patients with diseases caused by dysfunctional excitability are inadequately relieved of symptoms by existing drugs. For

example, up to 30% of patients with epilepsy do not achieve complete remission from seizures with the currently available medical treatments (Brodie et al. 2012), while others suffer from serious adverse effects (Sankar and Holmes 2004). Kv7 channels are good targets for drug development since they are involved in various kinds of diseases.

We have previously shown that PUFA and PUFA analogs can activate Kv7 channels (Liin et al. 2015, Liin et al. 2016). PUFAs are naturally occurring lipids formed by a carboxyl head group and a hydrocarbon tail. A PUFA has an unbranched hydrocarbon tail that contains two or more double bonds. For naturally occurring PUFAs, the double bonds are in *cis* conformation. A fatty acid containing one double bond is called a monounsaturated fatty acid (MUFA), while a fatty acid with no double bonds is called a saturated fatty acid (SFA). PUFA exist in several versions with a different number of carbons in the tails and a varying amount and position of double bonds. Certain fatty acids have names, although there are also general ways of naming fatty acids by describing their molecular properties, e.g. arachidonic acid which contains 20 carbons and four double bonds in its tail can be called 20:4 (ω -6) or 20:4 (5,8,11,14). 20:4 indicates 20 carbons and four double bonds in the tail, and (ω -6) the position of the first double bond (the omega double bond), counting from the methyl end of the tail. The alternative (5,8,11,14) indicates the position of each double bond, counting from the carboxyl end of the molecule. Some of the fatty acids used in this thesis are presented in figure 6A.

PUFA analogs are compounds that share a similar structure to PUFA, but the carboxyl head group is exchanged for something else, for example, an amino acid. There are many possible combinations of PUFA analogs. Certain PUFA analogs can be found endogenously in the body, for example, the endocannabinoid *N*-arachidonylethanolamine (Anandamide or AEA) consisting of an arachidonic acid tail with an ethanolamine head (Figure 6B) (Devane et al. 1992).

Figure 6. Selection of fatty acids and PUFA analogs used in this thesis.

PUFAs are part of the human diet and cannot be formed *de novo* in the human body. Certain PUFAs can be transformed in the body to generate other desired PUFA species.

Triacylglycerols and phospholipids are formed by PUFA, MUFA and SFA esterified to a glycerol or a phosphate group. Both triacylglycerols and phospholipids are abundant in the human body as an energy reserve (triacylglycerols) or as part of cell membranes (phospholipids). PUFA can also exist as free fatty acids that can be transported in the blood bound to albumin. Free PUFA, often generated from the hydrolysis of phospholipids by phospholipases, can interact with membrane proteins or be transformed into second messengers by oxygenases such as cyclooxygenase, lipoxygenase or cytochrome P450 epoxygenase. The oxygenases form eicosanoids, an important group of fatty acid metabolites that include, for example, prostaglandins, leukotrienes and thromboxanes.

There have been a number of studies on the beneficial effects of PUFA on cardiac and neuronal disorders (McLennan et al. 1988, Billman et al. 1994, Billman et al. 1997, Billman et al. 1999, Xiao and Li 1999, Marchioli et al. 2002). An extensive number of articles have also presented the effects of PUFAs on various voltage-gated ion channels. A large amount of data were reviewed in a 2017 paper (Elinder and Liin 2017). To generalize, it is observed that PUFA can inhibit Nav and Cav channels (Vreugdenhil et al. 1996, Tigerholm et al. 2012) and potentiate Kv channels (Borjesson et al. 2008, Liin et al. 2015). This thesis will focus on the described effects on Shaker Kv and Kv7 channels.

1.6.1 The activating mechanism of PUFAs

In a series of papers by Börjesson et al. 2008–2011, the initial findings describing the effect and mechanism of PUFA on Shaker Kv were presented (Borjesson et al. 2008, Borjesson et al. 2010, Borjesson and Elinder 2011). This work was later developed when the effect of PUFA on Kv7 channels was studied (Liin et al. 2015, Liin et al. 2016, Liin et al. 2018). PUFA activate Kv7 channels by shifting the voltage dependence of the channel opening towards negative voltages (ΔV_{50}) and increasing the overall conductance ($\Delta G_{\max}$) of certain channels. For illustrations of the two effects, see figure 7A.

The activating mechanism of PUFAs and PUFA analogs is called the lipoelectric mechanism (Borjesson et al. 2008, Borjesson et al. 2010, Borjesson and Elinder 2011). To summarize, this mechanism suggests that the lipophilic tail of the compound enters the cell membrane and moves close to the channel. The negatively charged head group remains in the interface between the extracellular fluid and the outer leaflet of the cell membrane, interacting with the channel in the border between the cell membrane and the channel. The negative charge of the head group attracts the positively charged residues in the extracellular parts of the channel. This attraction facilitates the activation of the channel. The opposite occurs if a positively charged PUFA analog is added. Then, the compound exerts a repulsive force on positively charged residues of the channel. The opposite effects of changed head charge polarity means that this mechanism can be used to tune the activity of the channel.

This section comprises a summary of some important findings that lead to the proposed mechanism. Docosahexaenoic acid (DHA) activates the Shaker Kv channel by shifting the voltage dependence of channel opening towards negative voltages (Xu et al. 2008). Important properties for enabling PUFA to activate Shaker Kv channels are two or more double bonds (i.e. polyunsaturated) in *cis* conformation (Borjesson et al. 2008). MUFA and SFA fail to activate the Shaker Kv channel, probably because of the lack of double bonds (Borjesson et al. 2008). Methyl esters of DHA that remove the charge of the head group are unable to activate the Shaker Kv channel, suggesting that the negatively charged head is essential for the effect (Borjesson et al. 2008). Increasing the pH of the extracellular solution to deprotonate DHA, thereby increasing the likelihood of the compound being negatively charged, causes the PUFA to induce a larger effect, also underscoring the importance of the

negative charge of the compound (Borjesson et al. 2008). Decreasing the pH can completely remove the effect of PUFA. Changing the head of the compound to be positively charged reverses the effect on Shaker K_v channels, making them open at more positive voltages (Borjesson et al. 2010). The top charges in S4 of the VSD have been shown to be important interaction partners for PUFA in Shaker K_v channels (Borjesson and Elinder 2011). In the Shaker K_v channel it has been shown that PUFA mainly act on the voltage dependence of the final movement of S4, a step that is strongly coupled to the opening of the channel pore (Borjesson and Elinder 2011).

A site of action on the Shaker K_v was first proposed in 2011 (Borjesson and Elinder 2011) and was subsequently further investigated in 2016 using molecular dynamics simulations (Yazdi et al. 2016). The suggested site is close to the VSD's third and fourth transmembrane segments on the lipid-facing side in the outer leaflet of the cell membrane. Experimental and simulation data agree on the suggested binding site.

For K_v7 channels, PUFA have been shown to activate $K_v7.1$ and $K_v7.2/3$ (Liin et al. 2015, Liin et al. 2016). The activation is similar to Shaker K_v , a shift of voltage dependence towards channel opening at lower voltages (Liin et al. 2015, Liin et al. 2016). It has also been reported that PUFA increase the overall conductance for $K_v7.1$ (Bohannon et al. 2020b). The negative charge of the PUFA head is also important for the effect on K_v7 channels (Liin et al. 2015, Liin et al. 2016). While PUFA can activate $K_v7.1$, it has been shown that PUFA analogs with a lower pKa have a significantly greater effect (Liin et al. 2015), most likely because the shifted pKa increase the likelihood of the compound being negatively charged at a physiological pH.

Naturally occurring PUFA did not activate $K_v7.1$ when it was co-expressed with KCNE1 (Liin et al. 2015). However, the PUFA effects could be restored if the pH of the extracellular solution was increased. Also, certain PUFA analogs with a lower pKa (e.g. N-arachidonoyl taurine (N-AT)) retained their effects on $K_v7.1 + E1$ in physiological pH (Liin et al. 2015). It is suggested that KCNE1 changes the local pH at the site of activation for PUFA, leading to protonation of the carboxyl head so that the PUFA become uncharged. However, it is not known how KCNE1 induces this change.

The top two arginines in S4 have been proposed as important for the PUFA's effect on $K_v7.1$ (Figure 7B&C). For $K_v7.1$, neutralization of R1 ($K_v7.1/R228Q$) removes the ability of DHA and N-AT to shift V_{50} , but retains N-AT's ability to increase G_{max} (Liin et al. 2015, Liin et al. 2018). In $K_v7.1 + E1$, R1 neutralization does not change the ability of N-AT to shift V_{50} . In contrast, the neutralization of R2 ($R231Q$) removes the effect of N-AT on V_{50} of $K_v7.1 + E1$ (Liin et al. 2018). N-AT retains its ability to increase G_{max} when R2 is neutralized.

The G_{max} effect has been linked to a different site at the pore of $K_v7.1$. Neutralizing a lysine (K326C) in S6 renders N-AT unable to increase G_{max} for $K_v7.1 + E1$. However, K326C does not disrupt the N-AT effect on V_{50} (Figure 7B&C) (Liin et al. 2018). Introducing a negative charge in position 326 by mutating to a glutamate (K326E) or using a cysteine-specific agent reversed the effect, making N-AT decrease G_{max} . It has been proposed that the effect of N-AT on G_{max} is due to an interaction between the negative charge of N-AT and the lysine. The attraction of the lysine to N-AT pulls the residue towards the lipid bilayer, leading to the increased ability of the selectivity filter to conduct potassium ions (Liin et al. 2018).

Since the effects on V_{50} and G_{max} are independent of each other and are linked to two different areas on the channel, it has been suggested that there might be two different binding sites for PUFA on $K_v7.1$. However, from our current data we cannot determine whether there is one

common or two separate binding sites. Unpublished molecular-dynamic simulations of PUFAs interacting with Kv7.1 suggest that there are two separate binding sites on Kv7.1 that correspond to the regions found to be important for each effect. One site is close to the top of S4 of the VSD and a second site is close to the top of S6 in the PD. Molecular dynamics data also suggest that the carboxyl head of PUFA has quite distinct positions for each binding site. Meanwhile, the PUFA tail forms several different interactions with the hydrophobic residues in the transmembrane segments of the channel, enabling a variety of positions.

It has recently been suggested that the effect of PUFA analogs could be controlled by changing the properties of the compound. By controlling the length of the fatty acid tail, the amount and position of double bonds in the tail, as well as changing the head group, the effect can be altered significantly (Bohannon et al. 2019, Bohannon et al. 2020b). This could potentially be used to control the effect of PUFA analogs in order to make them more selective towards a specific channel.

Figure 7. PUFA activate Kv7.1 by shifting the voltage dependence of channel opening towards negative voltages (ΔV_{50}) and increasing the maximum conductance (ΔG_{max}). (A) Illustration of the two effects of PUFA observed in a G(V) curve. Three illustrations, first on left the G(V) curve in which both effects are seen together, then two G(V) curves on the right in which the effects have been separated. (B) Schematic cartoon of the two effects of PUFA analogs on Kv7.1. (C) Channel model for Kv7.1 with residues important for the highlighted mechanisms (top and side view). The shift of V_{50} is dependent on the top arginines in S4 (R228 and R231) of the voltage sensor (red in the cartoon and model) (Liin et al. 2018). The effect of the maximum conductance (G_{max}) is dependent on a lysine in S6 (K326) of the pore domain (yellow in the cartoon and model) (Liin et al. 2018).

Despite several studies of the PUFA effects on Kv7 channels, there are still mechanistic questions that require answers. The impact of KCNE subunits on the effect of PUFA warrants further attention, particularly the mechanism for how KCNE1 abolishes the effect of PUFA on Kv7.1. Also, thus far, the PUFA mechanism has only been studied for Kv7.1. It would be interesting to study the PUFA mechanism for Kv7.2 and Kv7.3 in order to explore whether the activating mechanism is similar. To date, the effect of PUFA on Kv7.4 and Kv7.5 have not been reported. Thus, we do not know whether all Kv7 channels react in the same way to PUFA or if there is selectivity for certain channels. There is an evident need for additional studies of the effect of PUFA on Kv7 channels.

2. Aims of the thesis

The general aim of this thesis is to study unresolved aspects of the complicated interaction between Kv7 channels and PUFA, PUFA analogs and related compounds. We also aim to explore whether this compound group has potential as a medical treatment by studying new aspects of the effect of PUFA analogs, for example, to study whether PUFA analogs can activate Kv7 channels containing disease-causing mutations and the effect of PUFA analogs when used in combination with other compounds.

The specific aims are to:

1. Study the mechanism of PUFAs and PUFA analogs to understand structural determinants for the interaction between Kv7 channels and PUFAs, PUFA analogs and related compounds (Papers I, II and III)
2. Characterize disease-causing LQTS-associated mutations in Kv7.1 and study the ability to restore their function utilizing PUFA analogs (Paper I)
3. Explore the possibilities of PUFA analogs and related compounds to act more selectively by utilizing combined treatment with other activators (Paper III)

3. Methods

The methods section will primarily focus on the electrophysiological recordings upon which this thesis is based and the preparatory steps taken to ensure that the recordings functioned properly. The synthesis of compounds used in the experiments will not be described here. For further information, see the methods section of each article.

3.1 Mutagenesis

For Papers I–III, Kv7 channels have been point-mutated to study different aspects such as the mechanism of channel activation by PUFAs or the characterization of naturally occurring mutations associated with disease. The gene for each channel was in a plasmid (pGEM, pXOON, pXOOM) that also contained a T7 promotor and a gene for antibiotic resistance. The mutagenesis was performed using the Quikchange site-directed mutagenesis kit (Agilent Technologies). A pair of PCR primers were designed with slight mismatches to introduce the desired mutation in the gene. After running the PCR, the parental DNA was degraded using DPNI. The PCR product was transfected to competent cells (10XL-Gold ultracompetent cells, Agilent Technologies). Transfected bacteria were selected on antibiotic containing agar plates by expression of an antibiotic resistance gene. A single colony was selected for growth and the plasmid was purified using plasmid purification kits (e.g. PureLink HiPure plasmid Midiprep Kit, Invitrogen). The gene was sequenced to ensure the presence of the desired mutation. Correct plasmids were linearized and purified for transcription of capped RNA in vitro (T7 mMESSAGE mMACHINE kit, Ambion).

3.2 Preparation of oocytes and expression of ion channels

Oocytes from the African clawed frog (*Xenopus laevis*) were used in the experiments for this thesis. Oocytes were prepared in-house or bought from Ecocyte Bioscience, Dortmund, Germany. We obtained the in-house prepared oocytes through frog surgery. The surgery was performed in accordance with the recommendations of the Ethical Committee at Linköping University (Ethical permits #53-13 and #1941). The frog was anesthetized in a bath containing 1.4 g/l Ethyl 3-aminobenzoate methanesulfonate salt (Tricaine). The oocyte lobes were extracted via a small incision in the abdomen. The operation was non-terminal. The oocytes were separated from the surrounding connective tissue using a mixture of collagenases (LiberaseTM, Roche, Switzerland) diluted in a calcium-free solution (OR-2, in mM: 82.5 NaCl, 2 KCl, 5 HEPES and 1 MgCl₂; pH adjusted to 7.3 using NaOH).

The prepared oocytes were injected with a 50 nl cRNA solution (2.5–50 ng) using a Nanoject injector (Drummond Scientific, Broomall, PA). The prepared oocytes were stored (both before and after injection) in a Modified Barth's solution (MBS) supplemented with sodium pyruvate (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO₃, 15 HEPES, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 0.82 MgSO₄, 2.5 mM sodium pyruvate; pH was adjusted to 7.6 by NaOH). The oocytes were incubated for 1–4 days at 8–16° Celsius before being used for experiments.

3.3 Electrophysiology

The currents from ion channel-expressing oocytes were measured using the electrophysiological two-electrode voltage clamp technique (TEVC). TEVC is a technique that is used to artificially control the membrane potential of large cells such as oocytes (Stühmer and Parekh 1995). Two electrodes penetrate the cell membrane. One electrode measures the potential in the cell membrane and compares it to a reference electrode in an extracellular bath. The voltage is controlled by injecting current through the second electrode. The current that must be injected in order to set the correct voltage is recorded. The current is

used as an approximation of the currents produced by the channels in the cell membrane. The voltage can be set to change over time, thus enabling the measurement of currents at different voltages.

3.3.1 Setup

Most recordings were performed on a setup running a Dagan CA-1B Amplifier (Dagan, Mn, USA), Digidata 1440A digitizer (Molecular Devices, CA, USA), and pClamp 10 software (Molecular Devices, Ca, USA). The glass electrodes were filled with 3 M KCl solution and mounted on a silver-chloride wire. The electrode resistance was kept between 0.3–2 M Ω . Experiments were performed at room temperature (20–23°C). Currents were filtered at 500 Hz and sampled at 5kHz.

3.3.2 Bath solutions

The most common bath solution used during our experiments is called 1K, since it contains 1 mM of potassium. The exact content of 1K in mM are: 88 NaCl, 1 KCl, 0.8 MgCl₂, 0.4 CaCl₂, 15 HEPES. pH was adjusted to pH 7.4 using NaOH.

For certain experiments a bath solution with a high potassium content was used to change the reversal potential of potassium ions. The solution was called 100K and contained 100 mM potassium. The exact content of 100K in mM: 89 KCl, 0.8 MgCl₂, 0.4 CaCl₂, 15 HEPES. pH was adjusted to pH 7.4 using KOH.

For experiments with a different pH in the bath solution, the same 1K bath solution was used but the pH was set daily to the desired level using NaOH or HCl. Any remaining solution from the day was thrown away.

3.3.3 TEVC protocols

The TEVC protocols were of an overall similar design. The cell was kept at a holding voltage, usually -80 mV. The channels were activated by stepping to test voltages in 10 mV increments between -80 mV up to +70 mV (the exact range depended on the channel), followed by a tail voltage and then back to a holding voltage. The time spent at the test voltage and the tail voltage varied depending on what channel was being used, in general, 2 or 3 s test and 1 s tail (at -30 mV) for Kv7.1-5, in contrast to 5 s test and 5 s tail (at -20 mV) for Kv7.1 + E1. The highest test voltage measured depended on the voltage at which the channel had been fully opened. The start-to-start time also differed: 15 s start-to-start for Kv7.1-5 and 30 s start to start for Kv7.1 + E1.

For certain mutated channels that opened at very negative potentials, the holding voltage was set to more negative voltages or a pre-pulse was introduced before the test voltage to close the channel.

For measurements using arachidonoyl amine, a brief hyperpolarizing pulse (-120 mV for 5 ms) was introduced before the tail voltage to release the channels from inactivation.

For Paper III, a special protocol was used to study closing kinetics. This protocol goes from a holding voltage of -80 mV to an activating pulse to +20 mV (2 s), followed by a family of closing voltages between -140 to -20 mV in 10 mV increments.

3.4 Voltage-clamp fluorometry (VCF)

The principle behind VCF is that a fluorophore attached to the top of S4 experiences changes in the local microenvironment when S4 moves from its resting to its activated conformation. The fluorescence from the fluorophore can change if, for example, it approaches or moves away from a quenching residue. If the change in microenvironment alters the fluorescence of

the fluorophore, a change in fluorescence intensity is detected by the photodiode. This change in fluorescence is used as an indication of the movement of S4. The same change in fluorescence intensity can be detected if the movement of S4 moves a quenching residue closer to an immobile fluorophore. If the microenvironment around the fluorophore does not change even though S4 moves, there will be no change in the intensity of the fluorescence. Thus, the position of the fluorophore in relation to the rest of the protein is important and must be thoroughly tested.

For VCF measurements of Kv7.1, a cysteine was introduced at position 219 (Kv7.1/G219C) (Osteen et al. 2010). Oocytes were labeled for 30 minutes using 100 μ M Alexa-488-maleimide (Molecular probes, OR, USA), when the probe is attached the channel is referred to as Kv7.1/G219C* (+ E1), in which * indicates that the fluorophore is attached. An Olympus BX51WI upright microscope was used for VCF recordings. The light was passed through an Oregon green filter cube (41026; Chroma). The light was then focused through a 20 $\times$ water immersion objective onto the top of the oocyte. The returning fluorescence signal was focused on a photodiode and amplified using an Axopatch 200A patch clamp amplifier (Axon Instruments, CA, USA). Fluorescence signals were low-pass Bessel filtered (Frequency Devices, IL, USA) at 100–200 Hz, digitized at 1 kHz and recorded using pClamp 10 software.

The intensity of the fluorescence signal was obtained at different voltages in a similar manner to how the current amplitude was obtained in the TEVC recordings. Steady state intensity at different activating pulses was obtained during simultaneous recordings with TEVC. Overall, the protocols were similar to those used during ordinary TEVC.

3.5 Compounds

Compounds were usually obtained from Sigma-Aldrich (Stockholm, Sweden) or Cayman Chemical (MI, USA). The compounds were stored according to the manufacturers' recommendations. The compounds were usually delivered in Ethanol or dissolved in 99.5% ethanol in the lab.

Some compounds were synthesized in house by a chemist at Linköping University (Xiongyu Wu). An exact description of the synthesis is provided in the method section in Paper III. The compounds were dissolved in the lab in 99.5% ethanol.

Arachidonoyl amine (AA⁺) was generously provided to us by T. Parkkari from the University of Eastern Finland, Kuopio, Finland. For information regarding synthesis and handling, see Börjesson et al. 2010.

Compounds were diluted in a bath solution on the day of the experiments. For Papers I and II the concentrations are the expected effective concentration, 70% of the nominal concentration. This is based on data that suggest that PUFA binds to the chamber wall leaving 70% of the compounds free and capable of interacting with the oocyte. The experiments that predict 70% effective concentration are reported in Börjesson et al. 2008. Paper III reports actual concentrations, not effective concentrations.

The compounds were applied to the oocyte chamber using different methods. For Papers I and II an acute application of the compounds using a syringe was practiced. The volume was sufficient to replace the volume of the oocyte chamber multiple times. For Paper III the compounds were applied continuously using a peristaltic pump.

3.6 Data analysis

The data were analyzed using Clampfit 10.7 (Molecular devices, CA, USA) and GraphPad Prism 8 (GraphPad Software, CA, USA). When required, the data were compensated for oocyte leakage in Clampfit.

3.6.1 Estimation of G(V) and effects of compounds

Conductance was estimated from the tail currents. The current was measured immediately at the beginning of the tail. This is a reliable estimation of conductance if the kinetics of opening/closing is not too fast. Measurements at the beginning of the tail currents approximate the open probability of the channel after the activating pulse. To obtain the voltage dependence of conductance, the tail current is plotted against the corresponding voltage from the activating pulse. These values can be fit to a sigmoidal Boltzmann function (Eq. 1) to obtain the minimum amplitude (A_0), maximum amplitude (A_1), the voltage required to reach half the maximum conductance (V_{50}) and the slope (s) value. The $G(V)$ curves in this thesis are often normalized in order to enhance clarity.

$$G(V) = A_0 + (A_1 - A_0)/(1 + \exp((V - V_{50})/s)) \quad (\text{Eq. 1})$$

To estimate the effect of a compound, the values from a control run on the cell were compared to the test run on the same cell after the test compound had been applied.

A shift in the voltage dependence of opening was determined by calculating the difference in V_{50} . The estimated V_{50} from the control was compared with the corresponding V_{50} when the test compound was applied. The difference is referred to as ΔV_{50} .

In the same way, the maximum conductance from the Boltzmann curve can be used to calculate the difference in maximum conductance. A_1 corresponds to $G_{\max}$ when Eq.1 is used to fit a $G(V)$ curve. The $G_{\max}$ after application of the test compound was divided by $G_{\max}$ of the control run. The quota was used to calculate the percentage increase/decrease of $G_{\max}$ ($\Delta G_{\max}(\%)$).

These estimated changes were occasionally used to plot the concentration dependence or pH dependence of either effect. The effect (ΔV_{50} or $\Delta G_{\max}$) was then plotted as a function of the compound-/proton-concentration used at different measurements. To fit the data, we used a concentration-response equation (Eq. 2). From the fitted data we were able to extract an estimated maximum effect ($E_{\max}$), the concentration needed to reach 50% of the maximum effect (EC_{50}) and the Hill coefficient (N). N was for certain analyses set to 1.

$$\Delta E = E_{\max}/(1 + (EC_{50}/c)^N) \quad (\text{Eq. 2})$$

3.6.2 Estimation of time constants for kinetics of opening and closing

To estimate the effect on opening kinetics by N-AT in Paper I, we measured the time it took to reach 50% of the peak current of an activating pulse of +40 mV (after 3 s for Kv7.1 and 5 s for Kv7.1+E1). To estimate the effect on closing kinetics by N-AT in Paper I, we measured the time it took to reduce the amplitude (instantaneous tail current – current after 5 s of tail current to -20 mV) to 50%. The tail current was measured after a similar pulse that was used for the opening kinetics.

To estimate the effect on opening and closing kinetics by ARA-S in Paper III, we performed experiments in a high potassium solution. The first 500 ms of a current triggered by a depolarizing or hyperpolarizing pulse was fit to a single exponential function. The difference in τ in the presence and absence of ARA-S was used to determine the ARA-S effect on kinetics at different voltages.

3.6.3 Fluorometry analysis

The fluorescence obtained during steady-state activation was plotted against the corresponding voltage as Fluorescence–Voltage curves ($F(V)$). The $F(V)$ curves were then fitted with either a single Boltzmann (Eq. 1) curve for Kv7.1 or a double Boltzmann (Eq. 3) curve for Kv7.1 + E1. These Boltzmann fits provided the voltages required to reach half of the maximum of F (corresponding to V_{50} in Eq. 1, single Boltzmann) or $F1$ and $F2$ (double Boltzmann). The VCF recordings also generated current data that were analyzed as previously described.

$$F(V) = A0 + (A1 - A0)/(1 + \exp((V - F1_{50})/s)) + (A2 - A1)/(1 + \exp((V - F2_{50})/s)) \quad (\text{Eq. 3})$$

3.7 Statistical analysis

Data are presented as mean $\pm$ SEM. Generally, we repeated each experiment four or more times ($n \leq 4$). For statistical tests $P < 0.05$ was considered significant. The data from our experiments are assumed to be sampled from a Gaussian distribution. Thus, all data have been analyzed using parametric tests.

Statistics were used on several occasions in the papers for this thesis. When comparing the effects induced by two different compounds, we generally used an unpaired two-tailed t-test. An unpaired two-tailed t-test was also used when comparing the averages for control data with the averages for the test data. If we ran several t-tests simultaneously, we used Bonferroni correction to reduce the risk of a type 1 error.

When comparing multiple groups with a defined set of reference data, we used a one-way ANOVA with Dunnett's multiple comparison test. When comparing multiple groups against each other, we used a one-way ANOVA with Tukey's multiple comparison test.

4. Results and discussion

When I started working on this thesis it was known that PUFAs and PUFA analogs can activate Kv7 channels. The focus of my work has been to explore the activation of Kv7 channels by this group of activators to better understand their potential as medical treatments. In order to do so I have focused on both the mechanism for how PUFAs and PUFA analogs activate Kv7 channels as well as studying new aspects of the activating effect of PUFA analogs. The results and discussion section will be organized after the aims, presenting one aim at a time.

4.1 Aim I: Study the mechanism of PUFAs and PUFA analogs to understand structural determinants for the interaction between Kv7 channels and PUFAs, PUFA analogs and related compounds

Although the mechanism for PUFA activation of Kv7 channels has been studied in previous publications, there are still knowledge gaps regarding the structural determinants that impact the interaction between Kv7 channels and PUFA related activators. Most studies have been conducted on Kv7.1. Thus, there is a need to study the effect on other Kv7 channels. Also, for Kv7.1, there are further aspects that must be addressed, for example, the impact of beta subunits on the activating effect of PUFAs. This section will focus on insights into the mechanisms that have been uncovered during my thesis.

4.1.1 PUFA analogs affect S4 movement of Kv7.1 + E1

As stated in the introduction, PUFAs and PUFA analogs activate Kv7 by shifting V_{50} towards negative voltages and increasing G_{max} . It is also known that the PUFA analog N-AT accelerates S4 movement and shifts the voltage dependence of S4 movement towards negative voltages in Kv7.1 (Liin et al. 2015). However, the effect of PUFA analogs on S4 movement when Kv7.1 is co-expressed with KCNE1 was not tested. To explore the impact on S4 movement by PUFA analogs we used the VCF technique. For further information on the technique, see the methods section. It has previously been reported that during KCNE1 co-expression there are two movements of S4 indicated by two parts of a F(V) curve in a VCF recording (Barro-Soria et al. 2014). The first part of the F(V) curve is called F1 (at negative voltages) and correlates with the first S4 movement. The second part is called F2 (at positive voltages) and correlates with a second movement of S4 that occurs in the same voltage range as the pore opening. In Paper I, we tested the impact of PUFA analog N-AT on F1 and F2 to explore the impact of N-AT on S4 movement in Kv7.1 + E1.

The Kv7.1 channel used for these VCF recordings had a mutation that is used to attach a fluorophore (G219C) (Osteen et al. 2012, Barro-Soria et al. 2014). The channels also contained disease-causing mutations. Two mutated channels were used, one with a naturally occurring LQTS mutation in the top of S4 of the VSD (S225L) and one with a mutation in a LQTS position at the bottom of S6 of the PD (F351A). The disease-causing mutations will be described later in this Results and discussion section. The channels were co-expressed with WT KCNE1. N-AT shifted the voltage dependence of F1 and F2 for Kv7.1/G219C*/S225L + E1, as if both movements of S4 were shifted towards negative voltage (Figure 8A) (Paper I). For the other mutant (Kv7.1/G219C*/F351A + E1), it was not possible to deduce a similar F(V) curve due to difficulties with normalization because the channel opened at a very positive voltage and never saturated its opening in our recordings.

We also studied the kinetics of the two F(V) components. N-AT accelerated the kinetics of F1 in both mutants (Figure 8B) (Paper I). N-AT also accelerated the kinetics of F2 for

Kv7.1/G219C*/S225L + E1 and the kinetics of channel opening. For Kv7.1/G219C*/F351A + E1, the F2 component was shifted to very depolarized voltages making it hard for us to reliably quantify F2 kinetics. However, since the kinetics of channel opening was faster when N-AT was applied, it could suggest that F2 kinetics is also faster.

Figure 8. Mean $F(I)$ curve for Kv7.1/G219C*/S225L+KCNE1 in the absence (red symbols) or presence of 70 μ M N-AT (purple symbols). N-AT shifts the $F(V)$ curve towards negative voltages. (B) Kinetics of F1 before (red) and after (purple) N-AT application. N-AT makes the kinetics of F1 faster. Figures from Paper I.

To conclude, the data suggest that the PUFA analog N-AT shifts the voltage dependence of F1 and F2 to negative voltages and accelerates the kinetics of the change in fluorescence intensity when KCNE1 is present. This suggests that part of the activating mechanism of PUFA analogs is to facilitate the movement of S4, by shifting the movement of S4 towards negative voltages.

4.1.2 PUFA activation of Kv7.1 is modified by KCNE1

Our first publication regarding PUFA effects on Kv7 channels revealed that when Kv7.1 is co-expressed with KCNE1, the PUFA DHA is no longer able to activate the channel (Liin et al. 2015). We suggested that KCNE1 did not inhibit binding of PUFA to the Kv7.1 channel since the number of KCNE1 subunits in the Kv7.1 tetramer did not change the result. However, it was proposed that the presence of KCNE1 decreased local pH at the PUFA binding site and induced protonation of PUFA. Protonation caused the compound to lose its charge and become unable to activate the Kv7.1 + E1. The PUFA effects could be restored if the pH of the extracellular solution was increased. In the initial study, we presented pH dependence curves for the V_{50} effect of DHA for both Kv7.1 and Kv7.1 + E1. There was a clear 1 pH unit difference between the curves for Kv7.1 and Kv7.1 + E1 (Figure 9A) (Liin et al. 2015). PUFA analogs with a pKa of the head that was shifted to lower pH levels retained their effect on Kv7.1 + E1, as if these PUFA analogs were still negatively charged. The protonation theory was further reinforced by testing arachidonoyl amine (AA⁺), a PUFA analog that induces inhibition when positively charged by protonation. Inhibition of Kv7.1 by AA⁺ was potentiated in the presence of KCNE1, as if KCNE1 induces protonation of AA⁺. The mechanism by which KCNE1 induces protonation of PUFA was, however, not explored.

Understanding the mechanism for how KCNE1 induces decreased pH is important for predicting which PUFAs and PUFA analogs will be able to activate Kv7.1 + E1. To identify the structural determinants in Kv7.1 + E1 responsible for the PUFA protonation, we exploited the difference between Kv7.1 and Kv7.1 + E1 in the pH dependence of the V_{50} effect by DHA. In Paper II, we compared the pH dependence of the V_{50} effect by DHA for channels containing mutations introduced in motifs that could be responsible for the protonation of DHA to the corresponding curves on Kv7.1 and Kv7.1 + E1. In this work, we focused on charged amino acids in two regions, the N-terminus of KCNE1 and the S5-P-helix loop. Both regions contain several charged amino acids that could be attracting free protons.

Neutralization of the charged residues in the N-terminus of KCNE1 was achieved using three KCNE1 versions. All three versions expressed well and generated Kv7.1 + E1-like currents. The pH dependence of the DHA effect was tested by the application of 70 μ M DHA at different pH values. The data were plotted and fitted with a concentration-response curve. The pH dependence of the DHA effect for the mutant channels was then compared with the corresponding curves for Kv7.1 and Kv7.1 + E1 to see whether they had a Kv7.1 or Kv7.1 + E1-like curve. All the channels with alterations in the N-terminus of KCNE1 had a similar pH dependence of the DHA effect as WT Kv7.1 + E1 (Figure 9B) (Paper II). Removing the charged residues in the N-terminus of KCNE1 did not restore a Kv7.1-like pH dependence of the DHA effect, as if these extracellular charges are not important for the protonation of DHA in Kv7.1 + E1.

Figure 9. (A) pH dependence of the DHA-induced shift of V_{50} . ΔV_{50} values were normalized to the expected maximum ΔV_{50} based on a fitted concentration response equation. KCNE1 changed the pH dependence of the DHA-induced shift of V_{50} . There is around a 1 pH unit difference between the curves. (B) The same as A but with the addition of the corresponding data for the KCNE1 versions with removed extracellular charges as indicated in the figure. Removal of N-terminal charges in KCNE1 retained a Kv7.1 + E1-like DHA effect. Figures altered from Paper II.

We then turned to the S5-P-helix loop, an extracellular loop connecting the top of S5 to the pore helix. This loop is long and contains several negatively charged residues. The S5-P-helix loop has been shown to be a determining factor for the location of the G(V) on the voltage axis (Elinder et al. 2016). The loop has also been proposed to be capable of being in proximity to the top of KCNE1, based on disulfide bond studies (Xu et al. 2013). To test whether these charged residues could impact the protonation of DHA, we neutralized each negatively charged residue one by one to cysteines. When co-expressed with KCNE1, the Kv7.1 mutants generated channels that, in most cases, opened at slightly more positive voltages compared to WT Kv7.1 + E1 (Paper II).

By applying DHA we showed that the charge neutralization of residues in the loop generated channels with pH response curves for the DHA effect in between those of Kv7.1 and Kv7.1 + E1 (Figure 10). The shift in the pH dependence of the DHA effect from Kv7.1 + E1-like to Kv7.1-like was most prominent for Kv7.1/E290C+E1 (Paper II). The pH dependence of the DHA effect for Kv7.1/E290C + E1 overlapped the curve for Kv7.1. Accordingly, the apparent pK_a for the DHA effect was approximately the same for Kv7.1/E290C + E1 and Kv7.1. Since the amino acid cysteine is pH dependent, we also created Kv7.1/E290A. Kv7.1/E290A + E1 had the same pH dependence of the DHA effect as Kv7.1/E290C + E1. This ensured that the effect was not due to the pH dependence of the cysteine itself. The data suggested that the negative charges in the S5-P-helix loop promote protonation of DHA in Kv7.1 + E1, particularly E290.

Figure 10. Removal of negative charges in the extracellular S5-P-helix loop restored a Kv7.1-like DHA effect. The effect is partially restored for most constructs but completely for Kv7.1/E290C +E1. The pH response curve was normalized to the estimated maximum shift of V_{50} for each construct indicated in the figure. Figure altered from Paper II.

A potential mechanism for how these negative charges in the S5-P-helix loop could induce protonation of PUFA is that the loop is close to a PUFA binding site and attracts positively charged protons to this area. However, for this model to work, the negatively charged loop can only be in close proximity to PUFA when KCNE1 is present since the loop does not protonate PUFA when Kv7.1 is expressed by itself. If this is the case, the mutation E290C should not alter the pH dependence of the DHA effect in the absence of KCNE1. To test this, we expressed Kv7.1/E290C by itself and tested the effect of DHA. The pH-response curve for DHA and the apparent pKa for DHA were similar for Kv7.1/E290C and Kv7.1 (Figure 11A) (Paper II). This finding suggests that KCNE1 must be present in order for E290 to induce protonation of PUFA.

Figure 11. (A) Kv7.1/E290C has a Kv7.1-like effect of DHA, regardless of whether or not it is co-expressed with KCNE1, as if the mutation only changes the pH dependence of the DHA effect when KCNE1 is present. (B) Arachidonoyl amine (AA⁺) has a reduced ability to shift V_{50} of Kv7.1/E290C + E1 compared to Kv7.1 + E1, as if removing the negative charge in position 290 decreases the protonation of AA⁺. The molecular structure of AA⁺ is shown beneath the graph. (C) Restoring the negative charge in position 290 using cysteine-specific agent MTSES decreases the ability of DHA to shift V_{50} , as if Kv7.1/E290C-SES⁻ + E1 replicates the conditions in Kv7.1 + E1. Figures from Paper II.

To further test whether E290 changes the local pH we used AA⁺. It has been previously shown that AA⁺ has an increased effect on shifting V_{50} toward positive voltages when applied to Kv7.1 + E1 compared to Kv7.1 (Liin et al. 2015). The data were interpreted as if KCNE1 induced protonation of the amine head, which increased the electrostatic repulsion of S4. For Kv7.1/E290C + E1, we saw that AA⁺ induced a smaller effect on V_{50} compared to the effect on WT Kv7.1 + E1. The AA⁺ effect on Kv7.1/E290C + E1 was Kv7.1-like (Figure 11B)

(Paper II). This gave further support to the hypothesis that E290 is important for the KCNE1-induced change of local pH.

As a final test, we decided to study whether restoring the negative charge at position 290 in Kv7.1/E290C + E1 would revert the channel to having a pH dependence of the DHA effect similar to WT Kv7.1 + E1. For this we used a cysteine-specific agent called Sodium [2-sulfonatoethyl] methanethiosulfonate (MTSES⁻). It reacts and covalently attaches a negatively charged SES⁻ group to E290C. The attachment of SES⁻ did not change the biophysical properties of Kv7.1/E290C + E1 but reduced the shift of V₅₀ by 70 μM DHA at pH 8.2. After MTSES⁻ modification there was no significant difference between the effect of DHA on WT Kv7.1 + E1 and Kv7.1/E290C-SES⁻ + E1 (Figure 11C) (Paper II). This data provide support for the importance of the negative charge in position 290 for PUFA protonation.

4.1.3 Model for how KCNE1 induces protonation of PUFAs

In Paper II we proposed the following model: Kv7.1 expressed by itself can be activated by PUFA through a shift of V₅₀ in a G(V) curve towards negative voltages. PUFA activates Kv7.1 by interacting with S4 of the VSD. The negative charge of the PUFA head facilitates the movement of S4 by attracting the positively charged arginines in the top of S4 (Figure 12A). This facilitation of the S4 movement by DHA shifts V₅₀. When KCNE1 is co-expressed with Kv7.1, a conformational change is induced that relocates the S5-P-helix loop closer to the PUFA interaction site. The negative charges of the S5-P-helix loop (particularly E290) attracts free protons to the area leading to a decreased local pH. The decreased local pH protonates PUFA, rendering it uncharged and unable to activate the channel (Figure 12B). A Kv7.1-like pH dependence of the DHA effect of Kv7.1 + E1 can be achieved if the negative charges of the S5-P-helix loop are neutralized (Figure 12C).

Figure 12. Proposed model for how KCNE1 induces the protonation of PUFAs. (A) PUFA binds the channel in between two VSDs (green). The negatively charged head of PUFA attracts positively charged residues in S4 to facilitate voltage sensor movement. The negatively charged S5-P-helix loop is not close to the PUFA binding site. Thus, it cannot induce protonation of PUFA. (B) Co-expression with KCNE1 changes the conformation of Kv7.1 moving the S5-P-helix loop closer to the PUFA binding site. The negative charges of the loop attract protons to the PUFA binding site, lowering local pH. The lower pH protonates PUFA, rendering it unable to activate the channel. (C) Neutralization of the negative charges in the S5-P-helix loop reduce the ability of the loop to attract protons. The activating effect of PUFA is therefore preserved. Figure altered from Paper II.

In the Cryo-EM structure of *Xenopus* Kv7.1 (Sun and MacKinnon 2017), the S5-P-helix loop is located above the PD where it forms a negatively charged cap. This agrees with our results, suggesting that the loop is not close to the PUFA interaction site in Kv7.1, which is thought to be close to S4 of the VSD. No available structure of Kv7.1 + E1 was available during the writing of this thesis. However, molecular dynamics simulation data and experimental data

with cysteine bridges suggest that the S5-P-helix loop can be located close to the N-terminus of KCNE1 (Chung et al. 2009, Xu et al. 2013). This finding agrees with our proposed model.

The way in which KCNE1 induces the conformational change of Kv7.1 was beyond the scope of this study. From our data we can conclude that the neutralization of charged residues in the N-terminus of KCNE1 is insufficient to restore the PUFA effect. This suggests that the S5-P-helix loop is not directly attracted to KCNE1 by these charged residues. We propose that KCNE1 binding somehow induces a rearrangement of Kv7.1 that leads to the movement of the loop.

4.1.4 The activating mechanism of PUFA analogs for Kv7.2 and Kv7.3

The mechanism for the activating effect of PUFAs and PUFA analogs on Kv7 channels has primarily been studied on Kv7.1 and Kv7.1 + E1. We know that the first and second arginine (R1 and R2) in the top of S4 of Kv7.1 has been shown to be important for the shift of V_{50} by PUFA. The increase of maximum conductance by PUFA has been linked to a lysine in the top of S6 in the PD (Liin et al. 2018). The Kv7 channels are largely conserved in the amino acid positions that have been proposed to be important for the activation by PUFA. PUFAs shift V_{50} of the M-channel (Kv7.2/3) in a similar way to Kv7.1 (Liin et al. 2016). However, the mechanism of activation of Kv7.2/3 by PUFA has not been studied. We predict that it is likely that the activation mechanism for Kv7.2 and Kv7.3 by PUFA is similar to that for Kv7.1.

In Paper III, we studied homomers of Kv7.2 and Kv7.3 instead of heteromers of the channels. We decided to study homomers instead of heteromers to make it easier to explore the mechanism for each channel. Kv7.2 forms functional homomers when expressed, but the expression of Kv7.3 by itself gives rise to very small currents. To increase the current density of Kv7.3, a mutation was introduced in the PD (A315T). It is suggested that this mutation stabilizes the selectivity filter of Kv7.3 homomers to increase conduction and perhaps increase membrane expression (Gomez-Posada et al. 2010, Choveau et al. 2012a, Choveau et al. 2012b). Kv7.3/A315T will henceforth be referred to as Kv7.3^T.

We started by studying the top arginines in S4. We neutralized R1 and R2 in Kv7.2 and Kv7.3^T. The neutralization of R1 (Kv7.2/R198Q and Kv7.3^T/R227Q) caused the V_{50} to shift towards very negative voltages for both channels. This made it hard to close the channel properly. Neutralization of R2 (Kv7.2/R201Q and Kv7.3^T/R230Q) generates potassium channels that have lost their voltage dependence and are constantly open in a physiological voltage range. This has been previously shown (Miceli et al. 2008, Barro-Soria 2018). For Kv7.3^T/R230Q, it has been shown that the voltage dependence of opening is shifted to extremely negative voltages, not closing properly at -200 mV (Barro-Soria 2018). This made it impossible to estimate the effect of PUFAs or PUFA analogs. To shift V_{50} into a more physiological voltage range, an extra arginine was introduced three positions below R2 (Q204R in Kv7.2 and Q233R in Kv7.3). The same approach has been described for Kv7.1, which has the same problem (Liin et al. 2018). The introduction of the extra arginine was used for both mutants with neutralized R1 and R2, creating the following channels: Kv7.2/R198Q/Q204R, Kv7.2/R201Q/Q204R, Kv7.3^T/R227Q/Q233R and Kv7.3^T/R230Q/Q233R. The channels with the extra arginine expressed well, with V_{50} in a physiological range. We also created channels in which we neutralized the residue that corresponds to the conserved positive lysine (K326) in S6 that has been shown to be important for the increase of maximum conductance for Kv7.1 by PUFAs (Liin et al. 2018). This lysine in Kv7.1 corresponds to arginines in Kv7.2 and Kv7.3. The channels created for this purpose were Kv7.2/R291Q and Kv7.3^T/R330Q. See figure 13A for the positions of the mutations introduced to study the mechanism. To test whether these residues are important for

the activation of Kv7.2/3 by PUFAs, we used a PUFA analog called arachidonoyl-L-serine (ARA-S). ARA-S is an endogenous endocannabinoid-like compound. In Paper III we showed that it strongly activates the M-channel as well as Kv7.2 and Kv7.3 homomers. ARA-S will be discussed more thoroughly later in this Results and discussion section.

Neutralization of the first or second arginine in Kv7.2 (as Kv7.2/R198Q, Kv7.2/R198Q/Q204R, Kv7.2/R201Q/Q204R) decreased the ability of 10 μ M ARA-S to shift V_{50} towards negative voltages, as if both arginines are important for the effect (Figure 13B) (Paper III). The ability to increase maximum conductance did not change significantly (Figure 13C). The introduction of the extra arginine (Q204R) did not change the effect of ARA-S, as the effect on Kv7.2/R198Q and Kv7.2/R198Q/Q204R was equal. Meanwhile, neutralization of the S6 arginine decreased the ability of ARA-S to increase G_{max} (Figure 13C). The data for Kv7.2 fit well with the data for Kv7.1, suggesting that PUFA analogs activate homomeric Kv7.2 and Kv7.1 channels using a similar mechanism.

Figure 13. (A) Schematic side view of a Kv7 subunit. Amino acid sequences for hKv7.1, hKv7.2 and hKv7.3 are shown above and below the channel subunit. Amino acids mutated to study the mechanism of PUFA analogs are shown. The amino acids were selected based on similar experiments on Kv7.1 (Liin et al. 2018). **(B-C)** Neutralization of either of the top arginines in S4 of Kv7.2 decreases the ability of ARA-S to shift V_{50} . Neutralization of an arginine in S6 decreases the ability of ARA-S to increase G_{max} . **(D-E)** Neutralization of both top arginines in S4 of Kv7.3^T decreases the ability of ARA-S to shift V_{50} . Neutralization of an arginine in S6 has no effect on the ability of ARA-S to increase G_{max} . Figures altered from Paper III.

For Kv7.3, neutralization of either the first or the second arginine did not change the ability of ARA-S to shift V_{50} (Figure 13D). We therefore created a channel mutant in which we neutralized both R1 and R2 and introduced an extra arginine in position 233 (Kv7.3^T/R227Q/R230Q/Q233R). This channel expressed well and had a functional voltage gating in a physiological voltage range. Neutralization of both R1 and R2 together decreased the ability of ARA-S to shift V_{50} (Figure 13D) (Paper III). The fact that we were able to reduce the ARA-S effect by removing both arginines suggests that one of the arginines is sufficient for the full effect in Kv7.3^T. The neutralization of R330 did not alter the ability of PUFA to increase G_{max} (Figure 13E). However, the interpretation of the G_{max} effect in Kv7.3

might be complicated by the A315T mutation used for homomeric expression, since A315T is thought to stabilize the selectivity filter. It is known that the intrinsic open probability of the selectivity filter is an important factor for a PUFA analog's ability to increase $G_{\max}$ (Liin et al. 2018). The fact that these experiments were performed in the presence of the A315T mutation makes it harder to interpret the importance of R330 for the increase of $G_{\max}$ by PUFA analogs in the WT channel. However, in $Kv7.3^T$, R330 does not appear to be important for the increase of $G_{\max}$ by PUFA analogs.

To summarize, in Paper III we showed that for a PUFA analog, positively charged residues in the top of S4 are important for the shift of V_{50} in $Kv7.2$ and $Kv7.3$ and that a positive residue in S6 is important for the increase of $G_{\max}$ in $Kv7.2$. The mechanism shares important features with $Kv7.1$. The similarity of the effect to $Kv7.1$ is clearer for $Kv7.2$ than for $Kv7.3$. The effect of removing the basic residues in S4 and S6 is more pronounced for the effect of the PUFA analog N-AT on the $Kv7.1$ channel than for ARA-S effects on $Kv7.2$ and $Kv7.3$. The two effects of N-AT (ΔV_{50} and $\Delta G_{\max}$) could be almost completely abolished when the residues linked to each effect were neutralized in $Kv7.1$ (Liin et al. 2018). In comparison, the effect of ARA-S on $Kv7.2$ and $Kv7.3$ was instead reduced but still clear with about one half of the effect remaining. It could be speculated that the effect of the low-affinity compound N-AT (EC_{50} for the V_{50} effect by N-AT on $Kv7.1$ is 31 μM (Liin et al. 2018)) used for the studies on $Kv7.1$ could be easier to remove compared to the effect of ARA-S, which has a higher affinity (EC_{50} for the V_{50} effect by ARA-S on $Kv7.2/3$ is 7 μM) for $Kv7$ channels. However, more studies must be conducted to understand the difference.

4.2 Aim II: Characterize disease-causing LQTS-associated mutations in $Kv7.1$ and study the ability to restore their function utilizing PUFA analogs

As a second aim we were interested in studying the ability of PUFA analogs to be used as possible treatment for arrhythmias. For this purpose, we used a set of $Kv7.1 + E1$ channels containing mutations found in LQTS patients. We reasoned that it was important to test whether PUFA analogs would be able to activate channels containing mutations in different part of the channel. In Paper I, we selected and introduced eight known mutations that have previously been shown to express (Splawski et al. 1997, Bianchi et al. 2000, Yang et al. 2002, Yamaguchi et al. 2003, Henrion et al. 2009, Eldstrom et al. 2010, Harmer et al. 2010, Yang et al. 2013). The mutations were located in the VSD, PD, C-terminal domain and the KCNE1 subunit (Figure 14). As part of this study we also characterized the impact of these mutations on the biophysical properties of the channel.

Figure 14. Schematic side view of a $Kv7.1$ subunit and a $KCNE1$ subunit. The approximate position of the mutations studied in Paper I is marked in the cartoon. Figure altered from Paper I.

4.2.1 Mutations in $Kv7.1$ and $KCNE1$ alter biophysical properties

Six out of the eight mutations selected were actual LQTS mutations found in patients (in $Kv7.1$: F193L, V215M, S225L, R583C and in $KCNE1$: K70N and S74L). The final two

mutations studied in Paper I (In Kv7.1: L251A and F351A) were in positions in which LQTS-associated mutations have been found, but the actual LQTS-associated mutations (L251P and F351S) are known to not give rise to potassium current (Deschenes et al. 2003, Napolitano et al. 2005). These will therefore be referred to as LQTS-like mutations.

The Kv7.1 mutants were expressed by themselves and with KCNE1. KCNE1 mutants were only expressed as Kv7.1 + E1. The properties of the currents generated by the mutated channels were measured and compared to corresponding WT channels. Typical changes in the properties of the channels included a different voltage dependence of channel opening and altered kinetics of channel opening and closing (Figure 15A-C) (Paper I). A shift in voltage dependence of opening towards positive voltage, a slower channel-opening kinetics and a faster channel closing will all reduce the current produced by these mutated channels. For most mutants, the dysfunction induced by the mutations could at least be partially attributed to these altered biophysical properties (Table 1).

	Homozygous			Homozygous + E1			Heterozygous + E1		
	V ₅₀	T _{50,open}	T _{50,close}	V ₅₀	T _{50,open}	T _{50,close}	V ₅₀	T _{50,open}	T _{50,close}
Kv7.1 (WT)	-29.4 ± 1.4	48 ± 5.7	977 ± 59	+20.1 ± 3.2	1980 ± 710	967 ± 47	N/A	N/A	N/A
Kv7.1/F193L	-29.6 ± 0.4	<25	nd	+23.1 ± 1.1	1455 ± 760	483 ± 21	+29.5 ± 2.9	2082 ± 97	732 ± 51
Kv7.1/V215M	-25.2 ± 1.4	46 ± 5	499 ± 51	+47.5 ± 1.8	1755 ± 124	251 ± 12	+36.6 ± 1.6	2015 ± 53	512 ± 26
Kv7.1/S225L	+13.8 ± 1.5	269 ± 31	644 ± 57	+51.3 ± 2.7	2297 ± 697	458 ± 49	+35.0 ± 1.5	2191 ± 66	513 ± 51
Kv7.1/L251A	-3.2 ± 1.0	876 ± 102	3464 ± 157	+34.8 ± 8.3	2015 ± 224	766 ± 51	+14.3 ± 3.8	1525 ± 203	650 ± 121
Kv7.1/F351A	+34.7 ± 2.3	1113 ± 62	543 ± 40	> 50	nd	nd	+29.1 ± 5.2	1645 ± 68	572 ± 72
Kv7.1/R583C	-42.8 ± 2.3	nd	nd	+42.4 ± 3.2	1814 ± 124	588 ± 110	+44.6 ± 4.6	2414 ± 35	1073 ± 68

	Homozygous			Homozygous + Kv7.1			Heterozygous + Kv7.1		
	V ₅₀	T _{50,open}	T _{50,close}	V ₅₀	T _{50,open}	T _{50,close}	V ₅₀	T _{50,open}	T _{50,close}
E1/K70N	N/A	N/A	N/A	+43.0 ± 1.5	913 ± 102	166 ± 8	+25.5 ± 2.2	1659 ± 148	609 ± 49
E1/S74L	N/A	N/A	N/A	+41.8 ± 2.2	1784 ± 102	286 ± 7	+20.6 ± 4.6	1689 ± 141	517 ± 32

Shifted V₅₀ Slowed opening
 Accelerated closing

Table 1. Estimated midpoint of G(V) curve (V₅₀), time constant for kinetics of opening (T_{50, open}) and time constant for kinetics of closing (T_{50, close}) for indicated channels. Colored background of the box indicate that the biophysical function is significantly altered compared to WT. The significance of the alteration is tested with one-way ANOVA with Dunnett's multiple comparisons test comparing to corresponding WT channel. The heterozygous channels are compared to WT Kv7.1 + E1. Red indicate that V₅₀ is shifted towards positive voltages compared to WT. Green indicate that opening kinetics is slowed compared to WT. Blue indicate that closing is accelerated compared to WT.

For certain mutants, other alterations of biophysical properties were found. For Kv7.1/R583C, a pronounced inactivation was found; this inactivation caused the G(V) curve for Kv7.1/R583C to have a voltage dependence of opening shifted towards negative voltages compared to WT Kv7.1. The negative V₅₀ shift for Kv7.1/R583C compared to WT could, however, be removed by introducing a brief hyperpolarizing pulse before the tail current to release the channels from inactivation. Co-expression with KCNE1 suppressed the inactivation seen in Kv7.1/R583C. Since KCNE1 co-expression suppresses the inactivation of both WT Kv7.1 and Kv7.1/R583C, it could be speculated that the inactivation has a similar

mechanism for both WT Kv7.1 and Kv7.1/R583C and that the R583C mutation simply makes the inactivation more pronounced.

The F351A mutation induced the largest shift in V_{50} . Kv7.1/F351A + E1 shifted V_{50} towards positive voltages to an extent that we were not able to achieve voltages positive enough to saturate opening of the channel.

Figure 15. Example of biophysical properties altered by the LQTS mutations introduced in Kv7.1 + E1 in Paper I. (A) The mutations caused a shift in V_{50} towards positive voltages, seen here as the shift induced by S4 mutation S225L. (B) A slower opening kinetics was noted for certain mutations, seen here as normalized currents for Kv7.1 + E1 and Kv7.1/S225L + E1 in response to a depolarizing pulse from -80 mV to +40 mV. (C) A faster closing kinetics was noted for certain mutations, seen here as normalized currents for Kv7.1 + E1 and Kv7.1 + E1/K70N in response to a hyperpolarizing pulse from +40 mV to -20 mV. Figure altered from Paper I.

The most common form of these mutations in people is as heterozygous carriers. Heterozygous carriers have one mutated and one non-mutated allele. To copy the effect of the mutation in a heterozygous mutation carrier, we co-injected the mutated subunit with an equal amount of WT RNA (Kv7.1_{WT} + Kv7.1_{MUT} + KCNE1_{WT} or Kv7.1_{WT} + KCNE1_{WT} + KCNE1_{MUT}). This will be referred to as heterozygous injection. In general, heterozygous injections generated currents with more WT-like biophysical properties. The milder phenotype could be because the WT subunit is able to partly restore channel function in heteromers. However, based on our recordings, two separate populations of homomers or a disproportionate larger expression of the WT subunit cannot be ruled out.

4.2.2 Mutations in Kv7.1 alter biophysical properties by different mechanisms

TEVC recordings provide a lot of information on current properties, but limited knowledge regarding the mechanism by which the mutations alter biophysical properties. We predicted that the changed function of the channel induced by mutations could be due to altered VSD activation, changes in VSD to PD coupling or altered pore opening. To explore the underlying mechanisms, we selected two mutants that were further tested using VCF. We introduced S225L, a mutation of the VSD, and F351A, a mutation of the PD, in the channel used for VCF measurements. Both Kv7.1/G219C*/S225L and Kv7.1/G219C*/F351A showed G(V) curves shifted towards positive voltages compared to Kv7.1/G219C*. However, the F(V) curves displayed a clear difference, depending on which one of the two mutations it contained. For Kv7.1/G219C*/S225L, the F(V) and the G(V) curves were shifted to the same extent and were still overlapping the same as WT Kv7.1/G219C* (Figure 16A) (Paper I). In contrast, Kv7.1/G219C*/F351A had some degree of shift in the F(V) curve, while a larger shift in G(V), separating the curves (Figure 16B) (Paper I). When co-expressed with KCNE1, we saw differences in the way in which one of the two F(V) components, F1 or F2, was affected most. Kv7.1/G219C*/S225L + E1 had a major effect on F1, suggesting that S225L

mainly affects the initial movement of S4 (Figure 16C) (Paper I). In Kv7.1/G219C*/S225L + E1, F1 and F2 were hard to distinguish between. For Kv7.1/G219C*/F351A + E1, F2 shifted towards very positive voltages (Figure 16D) (Paper I). This made the difference between F1 and F2 clearer. By mainly shifting F2, we propose that F351A impairs coupling or makes pore opening harder.

Figure 16. Voltage-clamp fluorometry recordings from mutated Kv7.1 and Kv7.1 + E1 channels. Representative fluorescence traces and combined mean F(V)/G(V) curves for indicated mutant channels. The dashed lines represent corresponding measurements from Kv7.1/G219C* with and without KCNE1. (A&C) S225L mainly affects the first movement of the voltage sensor (F or F1), shifting the movement towards positive voltages. (B&D) F351A mainly shifts the opening of the pore to positive voltages, seen as a separation of the F(V) and G(V) curve in C and a shift to positive voltages of F2 in D.

To further explore these experimental results, we tried to reproduce our findings using kinetic models previously presented for Kv7.1/G219C* (Osteen et al. 2012) and Kv7.1/G219C* + E1 (Barro-Soria et al. 2014). The Kv7.1/G219C* model is a 10-state gating scheme (Figure 17A). The horizontal transitions indicate the main S4 movement that contributes to F1; the vertical transition indicates channel opening and the second S4 movement that contributes to the small F2 component. The Kv7.1/G219C* model allows for channel opening even though not all S4s are activated. This allows for the overlapping voltage range for F(V) and G(V). In contrast, the Kv7.1/G219C* + E1 gating scheme has six states (Figure 17B). The difference in the models is that KCNE1 limits channel opening to when only all four S4 are activated. This separates the F1 component from the F2 and G(V) curve. For further information about the models, see Paper I.

The effect of the mutations was reproduced by altering the models. To reproduce the data from Kv7.1/G219C*/S225L and Kv7.1/G219C*/S225L + E1, we shifted the voltage dependence of the main S4 movement 50 mV towards positive voltages. This shift in the kinetic model reproduced the experimental data, as if the S225L mutations mechanism was to impair the main S4 movement (Paper I). The experimental data for Kv7.1/G219C*/F351A and Kv7.1/G219C*/F351A + E1 were best reproduced by shifting the opening transition 140 mV towards positive voltages. This shift generated model data like the experimental data, as if the F351A mutation mainly affected the opening transition of the pore (Paper I). In total, VCF data and kinetic modelling both suggest that the mutations S225L and F351A in Kv7.1

induce altered biophysical properties through different mechanisms, in which S225L impaired S4 movement while F351A impaired a subsequent transition associated with channel opening.

Figure 17. Kinetic models for Kv7.1 and Kv7.1 + E1 channel gating. (A) A ten-state gating scheme for Kv7.1. Horizontal transitions represent the independent primary S4 movements that increase the fluorescence (generating F1). Vertical transition represents channel opening and secondary movement of S4 (generating F2). (B) A six-state gating scheme for Kv7.1 + E1. Horizontal and vertical movements represent the same transitions as for Kv7.1. The vertical gate-opening transition is however limited to only happen when all four S4 have completed the primary movement.

4.2.3 N-AT activates channels with mutations in either Kv7.1 or E1 regardless of mutation location and underlying mechanism

We hypothesize that if the reduced activity of channels containing LQTS-associated mutations could be restored to the activity of WT channels, the risk of arrhythmias would be reduced in LQTS patients. The activity of the channels could potentially be restored by Kv7 channel activators such as PUFAs or PUFA analogs. Since ordinary PUFAs are unable to activate Kv7.1 when co-expressed with KCNE1, we decided to test the ability of N-AT to restore the function of mutated channels. N-AT activates Kv7.1 + E1 by shifting voltage dependence of channel opening by up to -30 mV. This is a shift in the opposite direction compared to the shift induced by the LQTS mutations. We found that 70 μ M N-AT shifted V_{50} for all eight mutant channels by approximately -30 to -50 mV (Figure 18A) (Paper I). This N-AT-induced shift was about equal in size regardless of whether the channel was expressed as homozygous or heterozygous channels. As a functional test we quantified the amplitude of the current after a 5 s pulse to +20 mV and +40 mV before and after application of N-AT. N-AT increased the current amplitude of all mutated channels for both voltages tested. The largest effect on current amplitude was seen for the mutations that shifted the voltage dependence of channel opening the most. This is because these channels had the largest increase of open probability after N-AT application because +20 or +40 mV without N-AT only reached the foot of the G(V) curve. N-AT managed to compensate or over-compensate for the reduction of current induced by the mutation at these voltages (Figure 18B). N-AT also changed the kinetics of opening and closing by accelerating opening and slowing down closing, both reversing effects induced by the mutations on channel properties.

This data suggest that N-AT can be used as an activator of Kv7.1 + E1 channels containing mutations in various parts of the channel and with different mechanisms underlying their functional impairment. Since N-AT was able to restore the biophysical properties and increase the currents produced by the channels with LQTS mutations, it is likely that N-AT can be used as a general activator for Kv7.1 channels with a wide array of different channel mutations. However, this still requires the channel to be present in the cell membrane so it can be activated. Even though at least some of the impairment induced by the mutations studied in this thesis can be attributed to altered biophysical properties, we cannot rule out the possibility that that these mutations could induce trafficking problems in a human cell. The exact current amplitude in our experiments should probably be interpreted with caution.

Figure 18. (A) 70 μ M N-AT shifts V_{50} of $K_v7.1 + E1$ with mutations introduced in different parts of $K_v7.1$ or $KCNE1$. The ΔV_{50} is about the same, regardless of whether the channel is expressed as homozygous or heterozygous channels. In A, the dashed line denotes a mean shift in V_{50} for WT $K_v7.1 + E1$ upon N-AT application. # denotes an approximation. (B) An estimation of the ability of N-AT to restore current density for LQTS and LQTS-like mutants at +40 mV. The mean N-AT-induced increase in current amplitude of each mutant is multiplied by the control amplitude of each mutant. Data as mean $\pm$ SEM. In B, the dashed line denotes the relative current amplitude of WT $K_v7.1 + E1$ in the control solution (without N-AT). See Paper I for further details regarding B. Figures from Paper I.

4.3 Aim III: Explore the possibilities of PUFA analogs and related compounds to act more selectively by utilizing combined treatment with other activators

A problem with using K_v7 activators as a medical treatment is the similarity in structure of the K_v7 channels. This similarity makes it hard to develop drugs with selectivity for a specified K_v7 channel. For example, for retigabine, which activates all K_v7 channels apart from $K_v7.1$, it has been suggested that non-desired activation of $K_v7.4$ in the bladder could contribute to users experiencing urinary retention (Rode et al. 2010). This kind of adverse effect could be eliminated if treatments with better K_v7 selectivity could be developed. We hypothesize that a possible way of increasing selectivity would be to combine K_v7 activators with different mechanisms of activation and different selectivity for K_v7 channels. This combination could potentially be used to limit activation to certain K_v7 channels. To test this hypothesis, we used a group of endogenous PUFA analogs called endocannabinoids. This section will initially present the effect of endocannabinoids on $K_v7.2/3$ and then explore the possibility of combining endocannabinoids with other K_v7 activators.

4.3.1 Endocannabinoids can activate K_v7 channels

What initially sparked our interest in endocannabinoids was the structural similarities between endocannabinoids and previously found PUFA analogs that can activate K_v7 channels. Thus, in Paper III, we tested five endocannabinoids on the $K_v7.2/3$ channel. We found that the most common endocannabinoids – 2-arachidonoylglycerol (2-AG) and *N*-arachidonylethanolamine (Anandamide, AEA) – had no effect on $K_v7.2/3$ (Figure 19A-C); neither did *N*-arachidonoyl dopamine (NADA) (Figure 19A-C). However, two endocannabinoid-related compounds were able to activate $K_v7.2/3$, *N*-arachidonoyl-L-serine (ARA-S) and arachidonoyl- γ -aminobutyric acid (NAGABA) (Figure 19A-C). The largest effect was seen for ARA-S. The thesis will therefore focus on the details regarding the activation induced by this compound.

As described in section 4.1.4, ARA-S activated Kv7.2/3 in a similar way that PUFA analogs activated Kv7.1 and Kv7.1 + E1, namely, a shift in V_{50} towards negative voltages and an increase of $G_{\max}$ (Figure 19D) (Paper III). 10 μM ARA-S shifted V_{50} by -19.5 ± 1.5 mV and increased $G_{\max}$ by $53 \pm 7\%$. Both effects contributed to an increase of potassium current. The EC_{50} value was 7 μM for the shift of V_{50} with an estimated maximum shift of -31.9 ± 4.3 mV and 3 μM for the increase of $G_{\max}$ with an estimated maximum increase of $66 \pm 8\%$. By testing ARA-S-like compounds with small alterations to the head and tail, we studied the molecular determinants of the compound in order to activate Kv7.2/3. By changing pH to induce protonation and testing a non-charged version of ARA-S, we demonstrated that the negative charge of the head was essential for the activating effect. This fits well with previous data for PUFAs on Kv7 channels. The importance of the negative charge could also explain why 2-AG, AEA and NADA did not activate Kv7.2/3, as these compounds have uncharged head groups. Also, by testing several different tails we noted that different polyunsaturated tails and a monounsaturated tail with a serine head could activate Kv7.2/3. In contrast to previous studies, it appears that a single double bond of the endocannabinoid tail is enough to be able to activate Kv7.2/3, although not to the same extent as a polyunsaturated compound. A compound with a saturated tail and a serine head showed no effect on Kv7.2/3.

Figure 19. (A) Molecular structure of 2-AG, AEA, ARA-S, NADA and NAGABA. (B&C) Effect of 10 μM of 2-AG, AEA, ARA-S, NADA and NAGABA on Kv7.2/3. B shows the shift of V_{50} , C shows the change of $G_{\max}$. (D) Representative $G(V)$ curve for control and after application of 10 μM ARA-S. ARA-S shifts V_{50} towards negative voltages and increases $G_{\max}$. The dashed line is the $G(V)$ curve after application of 10 μM ARA-S normalized to the $G_{\max}$ of control. Figures from Paper III.

4.3.2 Improved Kv7 selectivity from combined treatments

To test the hypothesis that combining Kv7 activators could improve Kv7 subtype selectivity we would need activators with different underlying mechanisms and subtype selectivity. As previously mentioned in this thesis, ARA-S activated Kv7.2 and Kv7.3 using a similar mechanism to PUFA analogs activating Kv7.1. In Paper III, we also demonstrated that ARA-S does not share an activation mechanism with either retigabine or ICA73, by showing that mutations that remove the effect of retigabine or ICA73 did not change the effect of ARA-S. Since ARA-S activation of Kv7.2/3 is different from the activation by retigabine or ICA73, we thought that ARA-S could have a different subtype selectivity for Kv7 channels. We tested 10 μM ARA-S on all Kv7 members expressed as homomeric channels. ARA-S activated all Kv7 channels apart from Kv7.4 (Paper III). Kv7.1, Kv7.2, Kv7.3^T and Kv7.5 were all activated by a shift of V_{50} towards negative voltages and an increase of $G_{\max}$ (Figure 20A&B). In contrast, Kv7.4 was slightly inhibited by the ARA-S application, shifting V_{50} towards

positive voltages. This result is different from retigabine, which can activate Kv7.2 through Kv7.5 but not Kv7.1 (Figure 20C&D).

Figure 20. Effect of either 10 μM ARA-S (A&B) or 3 μM retigabine (RTG) (C&D) on the five isoforms of Kv7 channels expressed as homomers. (A&C) show the effect on V_{50} , (B&D) show the effect on G_{max} . ARA-S activates Kv7.1, Kv7.2, Kv7.3 and Kv7.5, and inhibits Kv7.4. RTG activates Kv7.2, Kv7.3, Kv7.4 and Kv7.5, and has no effect on Kv7.1. The dashed lines in A–D indicate the corresponding effect on Kv7.2/3 by indicated compound concentration. (E&F) Effect of 10 μM ARA-S, 3 μM retigabine (RTG) and co-application of 1 μM ARA-S and 1 μM RTG on Kv7.2/3, Kv7.1, Kv7.4, Kv7.5 and Kv7.4/5. E shows the effect on V_{50} , F shows the effect on G_{max} . Co-application of low concentrations of ARA-S and RTG decreases the undesirable activation of non-neuronal Kv7 channels and retains activation on Kv7.2/3. The dashed lines denote a shift in V_{50} of -20 mV (E) or an increase in G_{max} of 50% (F), which was the approximate effect of each treatment on Kv7.2/3. Figures altered from Paper III.

Since ARA-S had a different subtype selectivity than retigabine and ICA73, we hypothesized that a low concentration of ARA-S could be combined with a low concentration of either retigabine or ICA73 to retain the effect on the common target for both drugs, Kv7.2/3, while decreasing the effect on other Kv7 channels. To test this hypothesis, we initially compared the effect of a low concentration of ARA-S to a low concentration of retigabine, or a combination

of both, on Kv7.2/3. This test revealed that the co-application of both compounds had a significantly greater effect on both the shift in V_{50} and the increase of G_{max} compared to the effect of the low concentrations of each drug used separately (Paper III).

To further test this hypothesis, we tested the potential of the compound mixture to reduce the off-target effects on other Kv7 channels. We expressed Kv7.1, Kv7.4, Kv7.5 and heterotetramers of Kv7.4/5 and applied a high concentration of ARA-S (ARA-S_{high}, 10 μ M), a high concentration of retigabine (retigabine_{high}, 3 μ M) and the combination of ARA-S with retigabine (co-application, 1 μ M + 1 μ M). These concentrations were selected because they induced a similar V_{50} shift in Kv7.2/3. ARA-S_{high} induced large V_{50} shifts in Kv7.1, Kv7.5 and Kv7.4/5 as off-target effects (Figure 20E). Retigabine_{high} induced off-target effects on Kv7.4 and Kv7.4/5 by increasing G_{max} greatly (Figure 20F). Co-application succeeded in reducing all off-target effects induced by the higher concentrations of the compounds (Figure 20E&F) (Paper III). The shift of V_{50} for Kv7.1, Kv7.5 and Kv7.4/5 decreased to less than 4 mV and the increase of G_{max} for Kv7.4 and Kv7.4/5 was reduced to around 20% of the effect induced by retigabine_{high}. We also tested the concept by co-applying ARA-S with ICA73. This combination also decreased the off-target effects seen by high concentrations of each compound. Altogether, these experiments show that ARA-S in combination with either retigabine or ICA73 can be used to retain an activating effect on Kv7.2/3 and limit the off-target effects on other Kv7 subtypes.

4.4 Conclusions

My thesis explains several aspects of PUFA activation of Kv7 channels. Branching out from the initial work that studied the PUFA effects on I_{Ks} and the M-channel, I have been able to explain key mechanisms involved in PUFA-channel interaction. Below are some conclusions from my thesis:

For my first aim, we showed in Paper I that PUFA analogs facilitate the conformational change of the VSD by shifting the voltage dependence of the S4 movement towards negative voltages.

In Paper II, we presented an explanation of how KCNE1 co-expression induces the protonation of PFAs interacting with Kv7.1. KCNE1 induces a conformational change of Kv7.1, moving the S5-P-helix loop closer to the PUFA binding site. The S5-P-helix loop attracts protons to the PUFA binding site to reduce local pH in the site. The decreased pH protonates the PUFA, which loses its ability to activate Kv7.1.

In Paper III, we showed that the mechanism for the activation of Kv7 channels by PFAs and PUFA analogs is similar for Kv7.1 and Kv7.2 and, to some extent, Kv7.3. In contrast, the endocannabinoid ARA-S inhibits Kv7.4, suggesting that the mechanism is different for this channel.

For my second aim, Paper I presented how two mutations in different parts of the Kv7.1 channel cause dysfunction by different mechanisms. We also showed that, regardless of which parts of Kv7.1 or KCNE1 harbored the mutations, N-AT was able to activate the mutant Kv7.1 + E1 channels to restore much of the WT channel function. This suggests that N-AT can activate Kv7.1 + E1 channels with a broad range of dysfunctions as long as the channel is present in the cell membrane. In contrast to N-AT's ability to activate Kv7.1 + E1 channels containing mutations, other activators of Kv7.1 + E1 have shown varying effects on channels containing disease-causing mutations (Seeböhm et al. 2003, Xiong et al. 2007, Leitner et al. 2012). This suggests that the activating mechanism of N-AT might be better suited as a treatment option for LQTS patients.

For my final aim, Paper III reported that the combination of low concentrations of ARA-S in combination with retigabine or ICA73 activated Kv7.2/3 to a similar extent as high concentrations of each compound separately. The greatest benefit of the combination was the reduced activation of other Kv7 isoforms, most importantly, Kv7.1 and Kv7.4. This form of combination of Kv7 activators could prove beneficial as an alternative form of treatment if the development of selective Kv7 activators proves difficult.

5. Future directions

The findings presented in my thesis raise some important questions for the future. In this section, I provide some ideas for future projects.

To further evaluate the suitability of PUFA analogs as a medical treatment, additional tests of the effect on other channels found in nervous and cardiac tissue are required. There is also a need to test the effect of PUFA analogs on physiological systems such as cardiomyocytes, neurons or whole hearts. In two recent papers the effect of PUFA analogs has been tested on Nav, Cav, cardiomyocytes and guinea pig hearts (Bohannon et al. 2020a, Skarsfeldt et al. 2020). Bohannon et al. 2020 reported that the same PUFA analogs that can activate Kv7.1 + E1 can inhibit Nav and Cav channels. PUFA analogs inhibit Nav channels by shifting voltage dependence of inactivation and decreasing the maximum conductance. Cav channels are inhibited by a decrease in maximum conductance with no effect on the voltage dependence of inactivation. Certain PUFA analogs (e.g. DHA-glycine) were reported to be selective for the activation of Kv7.1 + E1 over the inhibition of Nav and Cav. DHA-glycine was found to shorten the action potential duration in human stem-cell derived cardiomyocytes and in a computational model of the heart's action potential. This contrasted with a non-selective PUFA analog which, in the computational model, resulted in an inability to elicit action potentials. The result of the non-selective PUFA analog suggests that it is important to consider the selectivity of PUFA analogs. In Skarsfeldt et al. 2020, DHA-glycine is used to shorten the QT interval in guinea pig hearts both *ex-vivo* and *in-vivo*, probably by activation of Kv7.1 + E1. The ability of DHA-glycine to shorten the QT interval could be beneficial as a treatment for LQTS syndrome in which prolongation of the QT interval creates the risk of life-threatening arrhythmias. In the future, it would be interesting to test whether PUFA analogs could offer protection from life-threatening arrhythmias in LQTS patients.

In Paper III, we presented some benefits of combining ARA-S with either retigabine or ICA73. One of the major benefits was an increased selectivity towards Kv7 channels found in neuronal tissue. However, we only presented data on cell models expressing a single type of ion channel. To further test the concept, additional studies would be required in more complex systems. Tests on neurons or animal models would greatly improve our knowledge of whether these compounds could be used as an antiepileptic treatment.

There are still questions regarding the mechanism for how PUFAs and PUFA analogs activate Kv7 channels. In Paper II, we used the difference between Kv7.1 and Kv7.1 + E1 in the pH dependence of the V_{50} effect by DHA. This difference was used to explain how KCNE1 co-expression induces protonation of PUFA. We proposed that the movement of the S5-P-helix loop closer to the PUFA binding site attracts protons that protonate PUFA. Preliminary data from our lab suggest there might be two separate binding sites for PUFA on Kv7.1, in which each site is responsible for either the shift of V_{50} or the increase of G_{max} . The G_{max} effect of DHA has a similar difference as the V_{50} effect in the pH dependence between Kv7.1 and Kv7.1 + E1 (Liin et al. 2018). It would be interesting to study how neutralization of the negative charges in the S5-P-helix loop would impact the pH dependence of the G_{max} effect of DHA on Kv7.1 + E1. This could reveal whether protonation of PUFA in both binding sites is explained by the reduction in local pH induced by the negative S5-P-helix charges, or if this is limited to only one of the PUFA binding sites. This could also provide additional information regarding the conformational changes induced by KCNE1.

In Paper III, we discovered that, rather than being activated by ARA-S, Kv7.4 was slightly inhibited. This suggests that the ARA-S effect on Kv7.4 is fundamentally different compared to the effect on the other Kv7 isoforms. This finding warrants further study in order to explore the difference in how Kv7.4 is affected by PUFAs and PUFA analogs.

6. Epilogue

This thesis is a continuation of the work initially started more than a decade ago. The combined work of the Elinder lab, Larsson lab, Liin lab and collaboration partners has made much progress regarding the mechanism for PUFA activation of K_v channels. We have been able to determine the molecular requirements for the compound in order to activate K_v channels, important interaction sites for the compounds on the channel and some initial data regarding the benefits of certain compounds on, for example, cardiomyocytes.

For my part, seven years have elapsed since I made my first recordings in Miami during what I then thought would be a six-month adventure but instead turned into a large chunk of my life. Throughout this time, my work in the lab has shifted from occasional nights and weekends, rotating part time with work at the hospital, to finally becoming a full-time job. I have learnt a lot and I have noted a lot of personal development over the years. As for my contribution to the field, only time will tell. I am proud of my publications and what we have investigated. Even though my study of PUFAs ends here, I am sure there will be many exciting findings in the years to come. I hope my contribution to the knowledge in this field will be useful when designing these future studies. I predict that in the next decade we will know a lot more about the potential of these compounds to be used as treatment.

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