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# Polyunsaturated Fatty Acids Modifying Ion Channel Voltage Gating

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Cover: Illustration of the proposed lipoelectric mechanism for polyunsaturated fatty acid-induced potassium channel opening. Top view of the  $K_v1.2/2.1$  channel with Shaker channel side chains showing the ion-conducting pore and one voltage-sensor domain. Residue R365 in orange and R362 in red.

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*To Tobias*



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## I. LIST OF PAPERS

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This thesis is based on the following papers, referred to by their Roman numerals:

- I. Xu XP, Erichsen D, **Börjesson SI**, Dahlin M, Åmark P, Elinder F. (2008) Polyunsaturated fatty acids and cerebrospinal fluid from children on the ketogenic diet open a voltage-gated K channel: a putative mechanism of antiseizure action. *Epilepsy Research* 80:57-66
- II. **Börjesson SI**, Hammarström S, Elinder F. (2008) Lipoelectric modification of ion channel voltage gating by polyunsaturated fatty acids. *Biophysical Journal* 95:2242-2253
- III. **Börjesson SI**, Parkkari T, Hammarström S, Elinder F. (2010) Electrostatic tuning of cellular excitability. *Biophysical Journal* 98:396-403
- IV. **Börjesson SI**, Elinder F. (2011) An electrostatic potassium channel opener targeting the final voltage-sensor transition. *Manuscript*



## II. ABSTRACT

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Voltage-gated ion channels play fundamental roles in neuronal excitability and therefore dysfunctional channels can cause disease. Epilepsy is such a disease, affecting about 1% of the population and being characterized by synchronous electric activity of large groups of neurons leading to various types of seizures. In this thesis, polyunsaturated fatty acids (PUFAs) were used as key substances to study a new pharmacological mechanism for how to induce opening of voltage-gated potassium ( $K_v$ ) channels, and how this possibly can protect against epileptic activity. All experiments were performed on cloned Shaker  $K_v$  channels expressed in *Xenopus laevis* oocytes. Channel activity was recorded with the electrophysiological two-electrode voltage clamp technique.

First we showed that both PUFAs and cerebrospinal fluid from children on the ketogenic diet open the Shaker  $K_v$  channel by shifting the channel voltage dependence towards more negative voltages, as we would expect for an antiepileptic effect. By testing fatty acids and related compounds with different properties and under different conditions we identified the critical structural components needed for the beneficial effect: a flexible *cis*-polyunsaturated lipid tail in combination with a negatively charged carboxyl head group. If substituting the negative charge for a positive amine group, channel opening was instead impeded. By mutating and modifying the channel at strategic positions the PUFA-action site was localized to a lipid-exposed surface close to the channel's voltage sensor. We also showed that PUFAs induce channel opening by electrostatically facilitating a final voltage-sensor movement. The PUFA efficiency is dependent on the channel's profile of charged residues in the outer end of the voltage sensor. This implies channel-specific effects. Finally, computer simulations demonstrated that small changes in channel voltage dependence can have dramatic effects on cellular excitability.

Both the identified PUFA-action site and the mechanism by which PUFAs induce channel opening are novel and could potentially be very useful in future drug design of compounds targeting neuronal and cardiac excitability. Our work also suggests that PUFA-induced  $K_v$  channel opening could be one important component in the ketogenic diet used as alternative epilepsy treatment.



# 1. INTRODUCTION

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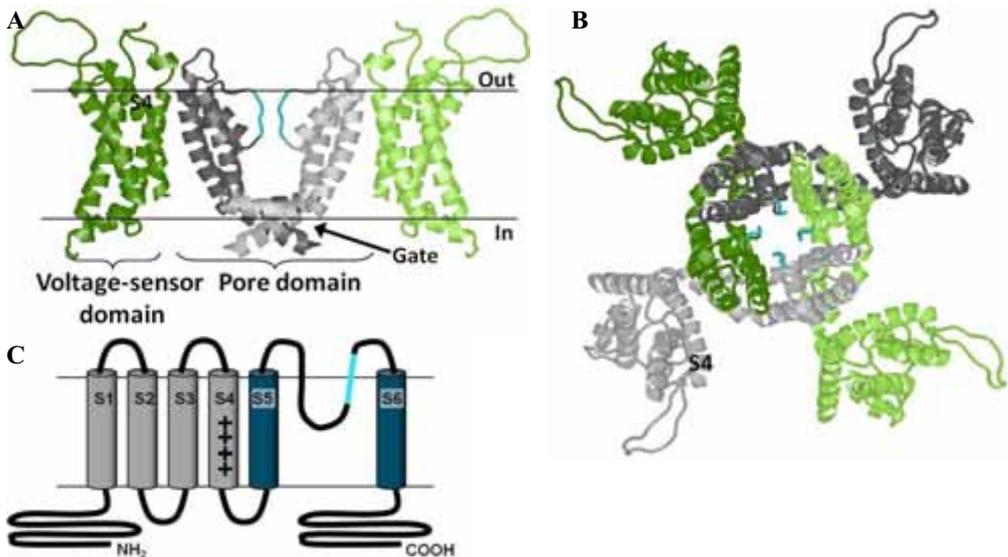
Neurons are the electrically excitable cells of the nervous system. They send electric messages in the form of action potentials. It was early recognized by Sidney Ringer that ions are critical for the electric activity of excitable cells (Ringer, 1882), and Julius Bernstein proposed that the resting membrane of a neuron is permeable to ions and that this permeability changes during excitation (Bernstein, 1902). Compound action potentials, measured from an intact frog sciatic nerve cell bundle, were first recorded in 1850 by Hermann von Helmholtz (Helmholtz, 1850; described in Haas, 1998), and single-cell action potentials recorded in frog myelinated axons by Edgar Adrian and Yngve Zotterman in 1926 (Adrian & Zotterman, 1926). At about the same time the first recordings of electric activity in the human brain, an EEG of an anesthetized boy, was made by Hans Berger (Berger, 1929). The hypothesized connection between ion permeability and membrane excitability was however not established until classical work in the 1930s, 40s and 50s were carried out. Alan Hodgkin and Andrew Huxley measured the intracellular neuronal action potential (Hodgkin & Huxley, 1939) and identified its main players:  $\text{Na}^+$ ,  $\text{K}^+$  and leak currents (Hodgkin & Huxley, 1952). In the 1960s and 70s voltage-gated Na and K channels generating these ion currents were finally, with help from toxins and other specific blockers, defined as separate units with water-filled pores (e.g. Tasaki & Hagiwara, 1957; Narahashi et al., 1964; Armstrong & Binstock, 1965). These early achievements were elegantly summarized by Bertil Hille in 1970 (Hille, 1970).

This thesis concerns pharmacological regulation of one of the main players in neuronal excitability: the voltage-gated potassium ( $\text{K}_v$ ) channel. I have studied a novel mechanism for how the activity of  $\text{K}_v$  channels can be modulated by charged lipophilic substances, and how this could protect against for instance epileptic seizures. Before discussing my experimental findings I will introduce the field of voltage-gated ion channels. I will briefly describe (i) the function of selected  $\text{K}_v$  channels in normal physiology, (ii) the role of  $\text{K}_v$  channels in disease, (iii) pharmacological strategies for regulating channel activity, and conclude by (iv) introducing polyunsaturated fatty acids (PUFAs) which are the key test substances in this thesis. The focus will be on  $\text{K}_v$  channels with occasional references to other channels.

## 1.1. $\text{K}_v$ channels – structure and principle of activation

All experimental work in this thesis is performed on the *Drosophila* Shaker  $\text{K}_v$  channel named after the hyperexcitable phenotype of flies with nonfunctional channels. It was the first  $\text{K}_v$  channel to be cloned (Kamb et al., 1987; Tempel et al., 1987; Pongs et al., 1988) and is therefore one of the most well-characterized.  $\text{K}_v$  channels are quite impressive by being only ~10 nm in diameter (Long et al., 2005a) but conducting more than 600,000 potassium ions per second when open (Armstrong, 1966; Hille, 2001). What do these fascinating membrane proteins look like? Today, the structure of  $\text{K}_v$  channels is quite well-known thanks to decades of experimental work that piece by piece have provided information about channel structure (Armstrong, 1975; Hille, 2001), lately confirmed by X-ray crystallography (Jiang et al., 2003; Long et al., 2005a; Long et al., 2005b; Long et al., 2007). Figure 1A-B shows the crystal

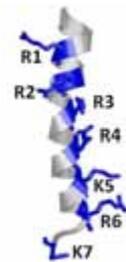
structure of an open  $K_v$  channel (the  $K_v1.2/2.1$  chimera (Long et al., 2007) with Shaker side chains, from Erik Lindahl, Royal Institute of Technology, Stockholm, Sweden). Four subunits (in different colours in Figure 1B) form the central pore domain flanked by the voltage-sensor domains. Each subunit consists of six transmembrane helices named S1-S6 (Figure 1C), where S1-S4 make up the voltage-sensor domains and S5-S6 the pore domain with the ion selectivity filter (cyan in Figure 1). This general architecture is also shared by voltage-gated Na ( $Na_v$ ) and Ca ( $Ca_v$ ) channels with the four subunits linked together into one single long protein molecule. The structure of voltage-gated ion channels has been described in detail in several reviews (e.g. Tombola et al., 2006; Catterall, 2010) and only selected components will be highlighted in this introduction.



**Figure 1.**  $K_v$  channel structure. The  $K_v1.2/2.1$  chimera with Shaker side chains in side view (A) and top view (B). Two voltage-sensor domains and two S5-S6 helices are removed for clarity in A. C) Schematic illustration of one  $K_v$  subunit. Selectivity filter marked in cyan in A-C.

### 1.1.1. Voltage sensitivity is controlled by mobile charges

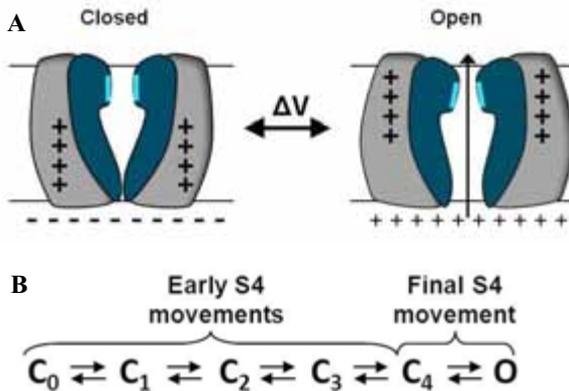
The voltage-sensor domains make the channel able to respond to changes in membrane potential. Already Alan Hodgkin and Andrew Huxley predicted that activation of a voltage-dependent channel must involve movement of charged particles (Hodgkin & Huxley, 1952). Today we know that these charged particles, called gating charges, are arginines and lysines in helix S4 which consequently is named the ‘voltage sensor’. The gating charge profile of the Shaker channel (arginines R1, R2, R3, R4, R6 and lysines K5 and K7) is seen in Figure 2 showing the well-conserved one-in-three pattern of positively charged residues (Börjesson & Elinder, 2008). Note also in Figure 1B that the voltage sensor is exposed to the surrounding lipid bilayer.



**Figure 2.** Gating charge profile of Shaker S4.

### 1.1.2. Channel opening involves several conformational steps

Most  $K_v$  channels are closed at negative voltages around the resting potential and activated at more positive voltages (Figure 3A). The positive voltage steps provide the energy needed for the voltage sensors to move from a ‘down’ position to an ‘up’ position. The outward movement of gating charges generates a small measurable current called the gating current (Armstrong & Bezanilla, 1973; Keynes & Rojas, 1974; Mannuzzu et al., 1996).  $K_v$  channel opening can be described by the simplified gating scheme in Figure 3B. The channel initially transits several closed states (denoted by C) when the voltage sensors move outward independently. When all four voltage sensors are in an activated up position ( $C_4$ ), there is a suggested concerted final S4 movement ( $C_4 \rightarrow O$ ), also called the opening step (Armstrong & Gilly, 1979; Keynes & Elinder, 1998; Schoppa & Sigworth, 1998; Pathak et al., 2005). The final voltage-sensor transition induces opening of the gate via the intracellular S4-S5 linker (McCormack et al., 1991; Long et al., 2005b), allowing ions to pass through the pore. Prolonged steps to positive voltages shut the channel by fast N-type inactivation or slow C-type inactivation depending on the design of the channel (Zagotta et al., 1990; Hoshi et al., 1991). Both fast and slow inactivation can reside in the same channel. The channel can also close (deactivate) by negative voltages that restore the down position of S4 and close the gate.



**Figure 3.** S4 movement during channel activation. **A)** Schematic illustration of the outward S4 movement induced by positive voltages. **B)** Simple six-state gating model for S4 movement during channel activation. (Figure 3B adapted from *Paper IV*)

Details of voltage-sensor movement are still debated due to the lack of a closed  $K_v$  channel crystal structure, as described in recent review papers (Tombola et al., 2006; Börjesson & Elinder, 2008; Catterall, 2010). Therefore, several alternative models for the closed channel structure and channel activation, based on different sets of experimental and computational data, exist. The main disagreements between different models concern the magnitude of S4 movement and whether S4 moves alone or together with helix S3. There is, however, a general agreement on the two most important points for this thesis: i) the S4s move outward during activation, and ii) a final voltage-sensor transition, possibly concerted, is needed for channel opening.

## 1.2. Voltage-gated ion channels are important for neuronal excitability

The human genome hosts 143 members in the superfamily of voltage-gated ion channels (Yu & Catterall, 2004). 40 of these are  $K_v$  channels divided into 12 subfamilies ( $K_v1$ -12). As a rule of thumb, opening of  $Na_v$  and  $Ca_v$  channels increase excitability while opening of  $K_v$  channels reduces excitability. For instance, the depolarization phase of the neuronal action potential (membrane potential rising to positive voltages) is generated by massive  $Na^+$  influx, and repolarization (membrane potential returning to negative resting voltages) by  $K^+$  outflux (Hille, 2001).  $Ca_v$  channels have a well-established function in the nerve terminal inducing  $Ca^{2+}$ -dependent transmitter release (Hille, 2001). The function of different ion channels in neuronal excitability has been extensively reviewed by others (e.g. Hille, 2001; Yu & Catterall, 2004; Vacher et al., 2008). Later in this thesis, the impact of experimentally observed  $K_v$  channel modulating effects on excitability will be discussed. In the following section, I will therefore briefly emphasize the function of these particular channels.

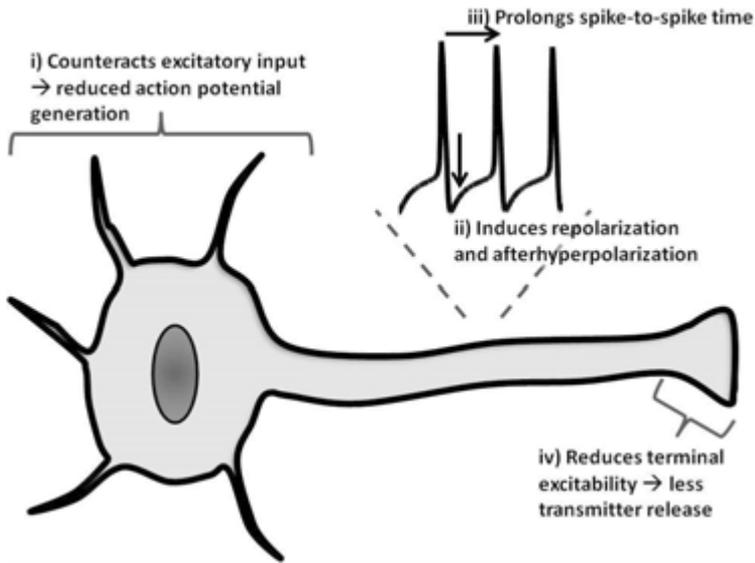
### 1.2.1. Delayed-rectifier and A-type channels dampen excitability

The Shaker channel can be considered to belong to two main subclasses of  $K_v$  channels, A-type channels or delayed-rectifier channels, depending on whether fast N-type inactivation is intact or not (Zagotta et al., 1990). This subclassification mainly refers to the appearance of the macroscopic  $K^+$  current where A-type channels display transient  $K^+$  currents with fast onset and fast inactivation (Connor & Stevens, 1971a), while delayed-rectifiers show persistent  $K^+$  currents with delayed onset and slow inactivation (Connor & Stevens, 1971b; reviewed in Baranauskas, 2007). Transient A-type channel currents are attributed to N-type inactivation. The mammalian counterpart to the Shaker channel is the  $K_v1$  subfamily which (as  $K_v$  channels in general) show impressive diversity by forming either homotetramers, heterotetramers with other  $K_v1$  subtypes, or complexes with auxiliary subunits (Vacher et al., 2008). For example, the homotetrameric  $K_v1.1$  channel is a delayed-rectifier channel but acts as A-type channel when coexpressed with  $K_v1.4$  or auxiliary  $\beta$ -subunits providing N-type inactivation. Other delayed-rectifier  $K_v$  channels mentioned in this thesis are  $K_v1.2$ ,  $K_v1.5$ ,  $K_v2.1$ ,  $K_v7.2$ , and  $K_v7.3$  while  $K_v4.1$  and  $K_v4.2$  are classical A-type channels. Opening of delayed-rectifier or A-type channels in different neuronal compartments (Vacher et al., 2008) reduce excitability as briefly described below (and schematized in Figure 4):

- i) A-type and delayed-rectifier channels make the resting potential in dendrites, soma, and axon initial segment more negative which counteracts excitatory input and prevents action potential generation (Hoffman et al., 1997; Jerng et al., 2004; Kole et al., 2007; Brown & Passmore, 2009).
- ii) Delayed-rectifier (and to some extent also A-type) channels shorten the action potential and induce afterhyperpolarization (membrane voltages more negative than normal resting potential) (Kang et al., 2000; Hille, 2001).

- iii) A-type and delayed-rectifier channels reduce action potential frequency by counteracting axonal depolarization and thereby prolonging the spike-to-spike interval (Kang et al., 2000; Hille, 2001; Guan et al., 2007).
- iv) A-type and delayed-rectifier channels prevent nerve terminal excitation and thereby neurotransmitter release (Geiger & Jonas, 2000; Dodson & Forsythe, 2004).

Note that this is a simplified description of how neuronal excitability is tuned by various  $K_v$  channels (the complexity is reviewed by Baranauskas, 2007).



**Figure 4.** Schematic illustration of delayed-rectifier and A-type channel effects on neuronal excitability.

### 1.3. Dysfunctional $K_v$ channels cause disease

The pivotal importance of voltage-gated ion channels in regulating excitability makes us vulnerable to dysfunctional channels that can cause diseases characterized by abnormal electric activity. Epilepsy is such a disease affecting about 1% of the population (Graves, 2006). The hallmark of epilepsy is synchronous electric discharges of large groups of neurons where the type and magnitude of seizure depends on which brain areas are affected. There are several lines of evidence of the involvement of delayed-rectifier and A-type channels in epilepsy as summarized in Table I: First, human  $K_v$  channel loss-of-function mutations are associated with different types of epilepsy syndromes. Second,  $K_v$  channel knockout in mice increases neuronal excitability. Third, pharmacological  $K_v$  channel block induces seizures. Dysfunctional  $K_v$  channels also cause disturbed cardiac function (Kaufman, 2009) and pain signalling (Cregg et al., 2010).

**Table I.** Example of K<sub>v</sub> channel modulations associated with epileptic activity.

Channel	Modulation	Syndrome	Effect	References
K <sub>v</sub> 1.1	Missense mutation	Episodic ataxia type 1 commonly associated with seizures	Faster deactivation and inactivation, impaired expression of WT K <sub>v</sub> 1.1	(Browne et al., 1994; Adelman et al., 1995; Zuberi et al., 1999).
K <sub>v</sub> 1.1	Knockout (mice)		Spontaneous seizures, reduced evoked seizure threshold, increased hippocampal excitability	(Smart et al., 1998; Lopantsev et al., 2003; Brew et al., 2007)
K <sub>v</sub> 1.2	Knockout (mice)		Spontaneous seizures, reduced evoked seizure threshold, increased hippocampal excitability	(Brew et al., 2007)
K <sub>v</sub> 4.2	Truncation	Temporal lobe epilepsy	Reduced current	(Singh et al., 2006)
K <sub>v</sub> 4.2	Knockout (mice)		Increased sensitivity to convulsant stimuli	(Barnwell et al., 2009)
K <sub>v</sub> 7.2	Missense mutation, deletion, truncation	Benign familial neonatal convulsions	Reduced current, accelerated deactivation	(Biervert et al., 1998; Singh et al., 1998; Singh et al., 2003)
K <sub>v</sub> 7.2	Heterozygous knockout/conditional knockout (mice)		Reduced seizure threshold/ spontaneous seizures	(Watanabe et al., 2000; Peters et al., 2005)
K <sub>v</sub> 7.3	Missense mutation	Benign familial neonatal convulsions	Reduced current, accelerated deactivation	(Charlier et al., 1998; Singh et al., 2003)
A-type	Pharmacological block (PiTx-K $\alpha$ )		Limbic and tonic-clonic seizures (mice)	(Juhng et al., 1999)
Delayed rectifier	Pharmacological block (TsTx-K $\alpha$ )		Limbic and tonic-clonic seizures (mice)	(Juhng et al., 1999)
Various K <sub>v</sub>	Pharmacological block (TEA, 4-AP)		Spontaneous seizures (rat hippocampal slices), Generalized seizures (rat)	(Fragoso-Veloz et al., 1990; Rutecki et al., 1990)

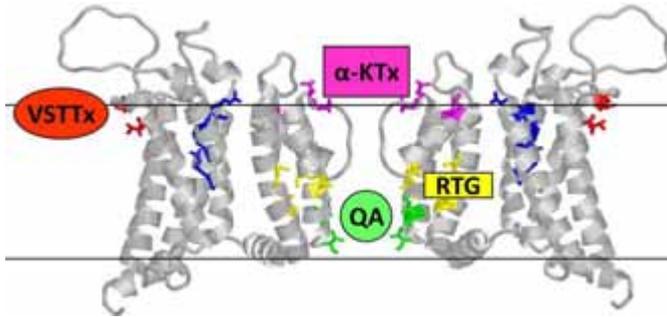
## 1.4. Ion channels as pharmacological targets

Irrespective of the underlying pathophysiological mechanism of disturbed excitability, a common and traditional strategy of treatment is to target various ion channels. In fact, ion channel drugs are the second largest group among approved drugs, only surpassed by those acting on G-protein coupled receptors (Mathie, 2010).

### 1.4.1. Pore block as traditional pharmacological mechanism

Most antiepileptic drugs act by reducing excitatory currents. The traditional pharmacological mechanism for antiexcitable drugs is to bind to the channel's pore, stabilize the inactivated channel conformation (although lately challenged, Nilsson et al., 2008) and thereby block ion

currents (Hille, 1977; Ragsdale et al., 1996; Rogawski & Loscher, 2004). This is the mechanism by which the antiepileptics carbamazepine, lamotrigine and phenytoin act on Na<sub>v</sub> channels (Ragsdale et al., 1996; Lipkind & Fozzard, 2010). The same binding site is also used by other quaternary ammonium compounds like local anaesthetics and class I antiarrhythmics blocking Na<sub>v</sub> channels (Ragsdale et al., 1996; Sheets et al., 2010) and K<sub>v</sub> channels (Zhou et al., 2001) (binding site in green in Figure 5). Pore block is also the mechanism of various toxins like tetrodotoxin and charybdotoxin, binding to the extracellular entrance of Na<sub>v</sub> and K<sub>v</sub> channels (magenta in Figure 5) (Hille, 1966; MacKinnon et al., 1990; Terlau et al., 1991).



**Figure 5.** Previously described binding sites for substances targeting K<sub>v</sub> channels. Binding sites for pore-blocking toxins (magenta), voltage-sensor domain-targeting toxins (red), retigabine (yellow), and quaternary ammonium ions (green) in the K<sub>v</sub>1.2/2.1 structure with Shaker side chains. Gating charges shown in blue. (Figure adapted from *Paper IV*)

#### 1.4.2. K<sub>v</sub> channel openers as new antiepileptic drugs

Instead of blocking excitatory ion channels, another potentially antiexcitable strategy is to enhance the activity of K<sub>v</sub> channels. K channel openers have been used since the 1990s on ATP-activated K (K<sub>ATP</sub>) channels to prevent the ischemic heart from intracellular Ca<sup>2+</sup> overload and to induce vasodilation during hypertension (Jahangir & Terzic, 2005). However, openers of K<sub>v</sub> channels have traditionally been surprisingly absent. The past decade has seen increased interest in K<sub>v</sub> channel opening as antiepileptic mechanism with focus on K<sub>v</sub>7 channels. The leading compound in the pipeline is retigabine (Rostock et al., 1996), which activates neuronal K<sub>v</sub>7.2-5 channels (Main et al., 2000; Rundfeldt & Netzer, 2000; Wickenden et al., 2000). Since retigabine, additional neuronal K<sub>v</sub>7 channel openers have been presented (Xiong et al., 2008; Fritch et al., 2010). A common feature of several of these (e.g. retigabine, acrylamides, zinc pyrithione) is to bind to specific residues in the pore domain and thereby keep the gate open (retigabine binding site in yellow in Figure 5) (Bentzen et al., 2006; Xiong et al., 2007; Lange et al., 2009). Open channel stabilization is manifested as negative shifts of the voltage dependence of channel activation, and in some cases increased current amplitude. Retigabine has successfully completed Phase III trial and is presently awaiting approval as an antiepileptic.

#### 1.4.3. The voltage sensor as an unexplored pharmacological target

Despite the great number of antiepileptics on the market, about 20-30% of patients with epilepsy respond incompletely to drug treatment (Lefevre & Aronson, 2000; Sillanpaa & Schmidt, 2006; Schuele & Luders, 2008). This patient group is classified to have pharmacoresistant or intractable epilepsy. Furthermore, many antiepileptic compounds are associated with adverse effects, including cognitive impairment of, for instance, learning and

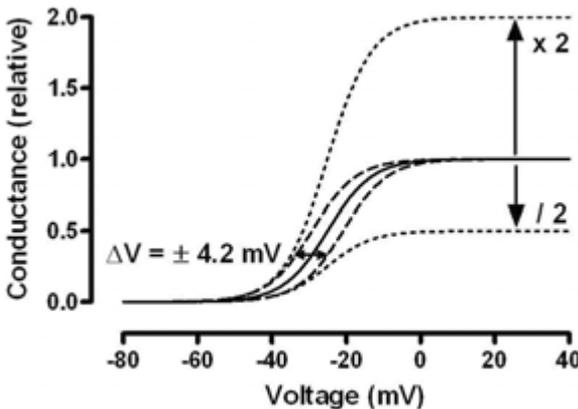
memory formation (Sankar & Holmes, 2004; Loring et al., 2007). The benefit of reduced seizure frequency therefore has to be balanced against negative side effects.

Lack of therapeutic effects in many patients in combination with adverse effects, motivates a constant search for new antiepileptic substances and new pharmacological mechanisms. The voltage-sensor domain has been suggested as an attractive, but so far unexplored, pharmacological target (Börjesson & Elinder, 2008; Catterall, 2010), evolutionary targeted by voltage-sensor trapping toxins binding to  $K_v$  and  $Na_v$  channels (red in Figure 5) (Swartz & MacKinnon, 1997; Swartz, 2007). One obvious reason is that the voltage-sensor domain, as a novel target, could provide antiepileptics that may be effective in cases resistant to existing treatment. Also, compounds binding to the voltage-sensor domain could have two theoretically advantageous features:

1) Voltage-sensor domain-targeting drugs have the interesting possibility to directly interfere with voltage-sensor movement and thereby affect channel voltage dependence. This could be via mechanic or electrostatic interactions. As schematized in Figure 6 (and discussed in Börjesson & Elinder, 2008), even small changes in the voltage dependence can have large effects on excitability. A shift in the voltage dependence of the schematic  $K_v$  channel conductance curve of only  $\pm 4.2$  mV is functionally equivalent to increasing or decreasing the number of  $K_v$  channels with a factor of 2. This is because of the steep voltage dependence of the  $K_v$  channels. The relationship between a shift in voltage dependence ( $\Delta V$ ) and the corresponding scaling factor of maximum conductance ( $f$ ) at voltages around channel opening, which is the region that matters for epileptic repetitive firing, is described as:

$$f = \exp(-\Delta V / s), \quad (\text{Eq. 1})$$

where  $s$  is the slope factor of the curve (here 6 mV). In reality the corresponding factor to a certain shift is expected to be even larger due to the asymmetric activation curve with steeper slope at the base of the curve (Almers, 1978).



**Figure 6.** Comparison of shifts in the voltage dependence and changes in maximum  $K_v$  channel conductance. Schematic conductance curve described as  $A/(1 + \exp(-(V - V_{1/2})/s))$  where  $A$  is the amplitude,  $V$  the voltage,  $V_{1/2}$  the midpoint and  $s$  the slope of the curve. Continues line is the control curve with  $A = 1$ ,  $V_{1/2} = -25$  mV, and  $s = 6$  mV. (Figure adapted from Börjesson & Elinder, 2008)

2) Drugs interfering with voltage-sensor movement could potentially have a larger therapeutic interval compared to pore-blocking compounds by inducing milder maximum effects. While the theoretical maximum effect of a pore blocker is 100% block (which could have detrimental effects on normal electric activity), the maximum effect of a voltage-sensor modifier could be a certain shift in the voltage dependence. Thus, even high concentrations of a voltage-sensor modifier may be well-tolerated because of a saturating effect on the voltage dependence.

In contrast to the  $K_v7$  channel openers described above interacting with the gate, this thesis project has aimed at finding and exploring substances targeting the voltage-sensor domain of  $K_v$  channels.

## **1.5. The ketogenic diet reduces neuronal excitability**

PUFAs are the key substances in my thesis and I will finally briefly explain the rationale behind this. The starting point of the first paper in the thesis was the contact with clinicians at Astrid Lindgren Children's Hospital in Stockholm, Sweden, treating children with severe intractable epilepsy with a fat-rich ketogenic diet. Diet instructions as epilepsy treatment have a long tradition and was documented already during antiquity (Magiorkinis et al., 2010). One antiepileptic recommendation that later has gained scientific approval, was complete abstinence from food and drink. Starvation induces ketosis by switching from glucose to fatty acids as the source of energy, and ketosis is believed to be one critical event preventing seizures (Stafstrom, 2004). In 1921, Russell Wilder suggested that the ketosis should be mimicked by a diet with high levels of fat and low levels of carbohydrates and proteins, and that such a diet could substitute for fasting (Wilder, 1921). This was the birth of the ketogenic diet and the first report of its clinical use to prevent seizures. In the classical ketogenic diet, the ratio of fat compared to carbohydrates and proteins is 4:1 (or sometimes 3:1) which means that 90% of the dietary energy originate from fat while only 10% come from carbohydrates and proteins (Neal & Cross, 2010). There are numerous studies supporting the usefulness of the ketogenic diet as epilepsy treatment for children. Three review articles evaluating the ketogenic diet efficacy, based on a total of 29 studies from 1970 to 2005, suggest that about 16% of children on the ketogenic diet obtain complete seizure control while up to 55% achieve more than 50% seizure reduction (Lefevre & Aronson, 2000; Henderson et al., 2006; Keene, 2006). Even though the ketogenic diet has been used as an alternative epilepsy treatment since the 1920s, the mechanism of action remains puzzling and several theories including augmentation of the GABA and antioxidant systems have been put forward (Rho & Sankar, 2008). Unravelling the mechanism is not trivial considering the complex metabolic effects of the ketogenic diet, making multiple targets and mechanisms more plausible than one single "magic bullet".

### **1.5.1. PUFAs and ketone bodies are possible key molecules**

One striking effect of the ketogenic diet is that serum concentrations of ketone bodies and PUFAs increase dramatically (20-fold and 3-fold, respectively) (Fraser et al., 2003). Both ketone bodies and PUFAs have therefore been speculated to be important antiepileptic components. The few clinical studies of PUFA supplementation in patients with epilepsy have

reported ambiguous results, possibly due to low PUFA doses and short administration periods (Taha et al., 2010). Antiepileptic PUFA effects are however clearly supported by studies in animal and cell models where acute (Leaf et al., 2005; Taha et al., 2010) or chronic (Verkerk et al., 2006; Scorza et al., 2009) PUFA supplementation reduce neuronal and cardiac excitability. Direct ion channel effects are suggested on cardiac  $\text{Na}_v$  and  $\text{Ca}_v$  channels showing PUFA-induced reduction in channel activity (Leaf et al., 2005; Boland & Drzewiecki, 2008). The antiepileptic effect could theoretically also come from opening of  $\text{K}_v$  channels. PUFA effects on  $\text{K}_v$  channels are however less explored.

PUFAs and ketone bodies are lipophilic substances and could therefore theoretically partition into the lipid bilayer and from there have direct access to the voltage sensor. The close interaction between lipids and the voltage sensor in  $\text{K}_v$  channels was suggested early (Elinder et al., 2001b) and later shown in the crystal structure (Long et al., 2007) and molecular dynamics (Freites et al., 2005; Jogini & Roux, 2007; Wee et al., 2011). Both ketone bodies and PUFAs are charged at physiological pH and could therefore speculatively interact electrostatically with S4. The chemical properties in combination with antiexcitable effects on other ion channels made us hypothesize that PUFAs and/or ketone bodies could affect  $\text{K}_v$  channels, possibly by targeting the voltage-sensor domain, and that this could be part of the success of the ketogenic diet.

## 2. AIMS OF THE THESIS

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The introduction leads us to the general aim of my thesis which was to test if charged lipophilic substances can regulate cellular excitability by interacting with the voltage-sensor domain of  $K_v$  channels. This could possibly be a powerful novel pharmacological mechanism of action for treatment of conditions with disturbed excitability.

The specific aims were to:

- study the effect of PUFAs and ketone bodies on the Shaker channel (*Paper I*)

*Paper I* showed that PUFAs, but not ketone bodies, induce Shaker channel opening by changing the channel's voltage dependence. The subsequent specific aims were therefore to:

- elucidate the mechanism of action for PUFA-induced Shaker channel opening (*Paper II-IV*)
- identify the PUFA site of action on the Shaker channel (*Paper IV*)
- test if PUFA-induced  $K_v$  channel opening could contribute to the antiepileptic property of the ketogenic diet (*Paper I and III*)



### 3. METHODOLOGY

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Below follows a brief description of the main methods used in the thesis. The purpose is to provide a general understanding of each method. For more detailed information, please refer to the Method sections of each paper.

#### 3.1. Preparation of oocytes and channels

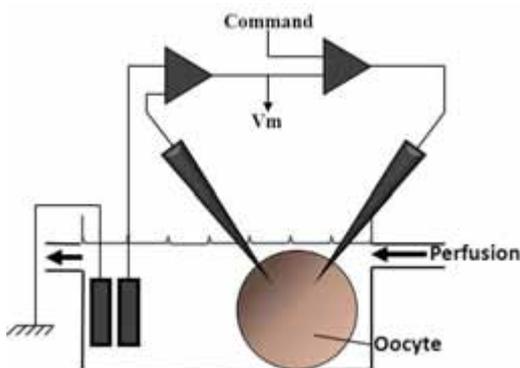
All experiments in this thesis are performed on cloned Shaker K<sub>v</sub> channels expressed in oocytes from the South African clawed frog *Xenopus laevis*. The ability of *Xenopus* oocytes to synthesize exogenous proteins upon injection of RNA was first described in 1971 (Gurdon et al., 1971) and has since then become a commonly used expression system to study ion channels (Miledi et al., 1982). The reason for its popularity is manifold. First, the *Xenopus* oocyte is large, up to 1.3 mm in diameter (Dumont, 1972), making it easy to handle and allowing insertion of intracellular electrodes for measurements. Second, the oocyte willingly synthesizes protein from injected RNA facilitating robust electrophysiological measurements with high signal-to-noise ratio. Third, the oocyte has few endogenous ion channels active in the voltage range normally covered during ion channel measurements (Wagner et al., 2000). Therefore, endogenous currents are usually negligible compared to currents produced by cloned channels. Thus, expression of cloned ion channels in *Xenopus* oocytes enables studies of a single type of ion channel without interference from the numerous other ion channels normally present in for instance a neuron.

The ovaries of adult female *Xenopus laevis* contain large amounts of oocytes organized in lobes in which oocytes at all developmental stages (I-VI) are distinguished (Dumont, 1972). Stage V-VI oocytes were selected for cRNA injection, characterized by the large size, distinct boundary between brown animal and yellowish vegetal hemisphere, and being enveloped by a vitelline layer (Dumont, 1972). Most experiments were performed on the wild-type voltage-gated Shaker H4 channel (Kamb et al., 1987) with residues 6-46 removed to disable N-type inactivation (Hoshi et al., 1990). Throughout this dissertation and in *Paper I-III* this channel is referred to as Shaker WT or simply Shaker, and in *Paper IV* as WT-IR. *Paper III* describes the approach for collecting and isolating oocytes. *Paper III* and *IV* describe how cRNA for WT and mutated channels was prepared and injected into oocytes. Animal experiments were approved by the local Animal Care and Use Committee at Linköping University.

#### 3.2. Electrophysiological measurements

During the 20<sup>th</sup> century, methods have been advanced by making it possible to measure the transmembrane flux of ion currents (Hodgkin & Huxley, 1952) and to study the behaviour of single ion channels involved in neuronal activity (Neher & Sakmann, 1976; Hamill et al., 1981). The electrophysiological method used in this thesis was two-electrode voltage clamp which is a common technique for measuring whole-cell channel currents in *Xenopus* oocytes (Stühmer, 1992). Two electrodes are inserted into the oocyte (Figure 7). One of them measures the voltage inside the oocyte relative to a reference electrode on the outside. The membrane voltage is clamped to a command value and can quickly be changed by injecting a

current into the oocyte with the second electrode. A voltage change inducing channel opening will increase membrane permeability and a current has to be injected to keep the voltage constant. The injected current is a direct measure of the channel current by being equal in size and kinetics. Channel voltage dependence is studied by measuring the current at different voltages.



**Figure 7.** Schematic illustration of the two-electrode voltage clamp technique used to measure ion and gating currents.

Most commonly the two-electrode voltage clamp technique was used to measure the ionic  $K^+$  current through the pore at different voltages. Steady-state currents were measured at the end of each test pulse (normally 100 ms long, ranging from  $-80$  to  $+50$  mV from a holding voltage of  $-80$  mV) and the conductance ( $G$ ) calculated according to Ohm's law:

$$G(V) = I_K / (V - V_{rev}), \quad (\text{Eq. 2})$$

where  $V$  is the absolute membrane voltage,  $I_K$  the steady-state  $K^+$  current, and  $V_{rev}$  the reversal potential of the  $K^+$  ion. The conductance was then plotted against the voltage to generate a conductance *versus* voltage,  $G(V)$ , curve. Substance-induced shifts of the  $G(V)$  curve along the voltage axis was the parameter used to quantify the effect of each substance. Shifts in either negative or positive direction along the voltage axis will be described simply as 'negative' or 'positive' shifts. The shift was measured at 10% of maximum conductance as described in *Papers I-IV*. This level was selected because it is less sensitive to alterations in the maximum conductance sometimes caused by endogenous currents and voltage-dependent block by intracellular ions. Statistical significance of induced shifts was generally tested with two-tailed  $t$ -test, or one-way ANOVA followed by multiple comparison tests if several groups were compared.

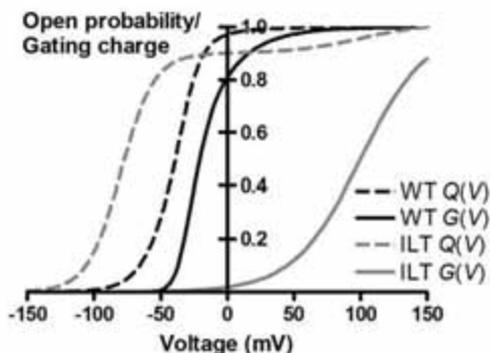
The concentration dependence of PUFA-induced shifts is expressed as:

$$\Delta V = \Delta V_{\max} / (1 + K_D/c), \quad (\text{Eq. 3})$$

where  $\Delta V_{\max}$  is the maximum effect,  $K_D$  the dissociation constant, and  $c$  the concentration of the compound.

To study voltage-sensor movements preceding channel opening we instead measured gating currents. The W434F pore mutation was introduced to prevent ion conductance (Perozo et al., 1993) unmasking the smaller gating current. The corresponding shift of the gating current *versus* voltage,  $Q(V)$ , curve was measured by plotting the integrated OFF gating current (the current generated by inward S4 movements when stepping to negative voltages) against the pre-pulse voltage (described in *Paper IV*), and sliding the control curve along the voltage axis until it overlapped the DHA curve.

To quantify PUFA effects on early S4 movements and the final S4 transition we used the Shaker ILT mutant (Smith-Maxwell et al., 1998). As seen in the model calculations in Figure 8, the early S4 movements (the major part of the  $Q(V)$ ) occur at very negative voltages in the ILT mutant while the final S4 movement (reflected in the  $G(V)$ ) is isolated to positive voltages. This creates a better separation between early and final S4 transitions compared to WT which shows partly overlapping voltages for the  $Q(V)$  and  $G(V)$  curve.



**Figure 8.** Comparison of the theoretical voltage dependence of early and final S4 transitions in WT and ILT mutated channels as described in *Paper IV*. (Figure adapted from *Paper IV*)

### 3.3. Test substances

Table II lists the test substances used in each paper of which the  $\omega$ -3 PUFA docosahexaenoic acid (DHA) was most extensively studied. Throughout the papers and this thesis, the concentration of free fatty acids and related substances is corrected for substance binding to the experimental chamber. As described in *Paper II*, about 30% of radioactive fatty acids bind to the experimental oocyte chamber independent of fatty acid concentration. Only about 70% of the added free fatty acids will thereby be available to affect the channel. The cyclooxygenase inhibitor indomethacin was used for arachidonic acid, arachidonic acid-me, and arachidonyl amine experiments to prevent substance metabolism.

**Table II.** Test substances used in *Papers I-IV*.

Test substance <sup>†</sup>	Type of substance		Charge <sup>‡</sup>	Paper			
		Carbons:double bonds		I	II	III	IV
Acetic acid	SFA	2:0	–		●		
Acetoacetic acid	Ketone body		–	●			
Arachidic acid	SFA	20:0	–		●		
Arachidic acid-me	Methyl ester	20:0	0		●		
Arachidonic acid	PUFA	20:4 ω-6	–		●	●	
Arachidonic acid-me	Methyl ester	20:4 ω-6	0			●	
Arachidonyl amine	Amine	20:4 ω-6	+			●	
Butyric acid	SFA	4:0	–		●		
DHA	PUFA	22:6 ω-3	–	●	●		●
DHA-me	Methyl ester	22:6 ω-3	0		●		
EPA	PUFA	20:5 ω-3	–	●	●		
GsMTx4	Toxin		++++		●		
Hexadecatrienoic acid	PUFA	16:3 ω-3	–		●		
DL-β-Hydroxybutyric acid	Ketone body		–	●			
Linoleic acid	PUFA	18:2 ω-6	–	●	●		
Linolenic acid	PUFA	18:3 ω-3	–		●		
Linolenic acid-me	Methyl ester	18:3 ω-3	0		●		
All-trans-Linolenic acid	PUFA	18:3 ω-3	–		●		
Oleic acid	MUFA	18:1 ω-9	–		●		
Oleic acid-me	Methyl ester	18:1 ω-9	0		●		

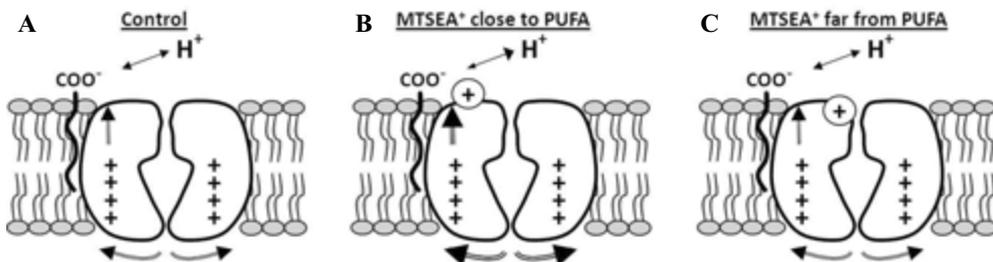
<sup>†</sup> as named in the thesis and articles. For full chemical names, see respective article. <sup>‡</sup> charge at pH 7.4 as expected from the pKa value. SFA: saturated fatty acid, MUFA: monounsaturated fatty acid.

### 3.4. Strategy to modify the degree of protonation

As described in *Paper II*, experimentally induced changes in the degree of DHA protonation was one of the strategies used to test the importance of the PUFA carboxyl charge. This was done either by changing pH of the extracellular solution or by introducing additional positive surface charges on the channel using mutagenesis and cysteine modification. Changes in protonation were also used in *Paper IV* to identify the PUFA site of action, shortly referred to as the PUFA-action site. The strategy is described thoroughly in *Paper IV* and briefly below.

Cysteines have a special place in ion channel research by being specific targets to methanethiosulfonate (MTS) reagents that form –SS– bridges with cysteines (Karlin & Akabas, 1998). These MTS reagents come in many different flavours, each with its specific chemical attribute. The property of a cysteine side chain can therefore be altered in numerous ways depending on the design of the MTS molecule. In *Paper IV* strategic residues were first point-mutated to cysteines and then modified with the small positively charged MTSEA<sup>+</sup> reagent (Figure 9). A modified residue close to the PUFA will increase the local pH, deprotonate the PUFA and thereby make it more potent (Figure 9B), while a modified residue far from the PUFA should not affect the PUFA potency (Figure 9C). For some mutants also the larger MTSES<sup>–</sup> reagent was used to test residue accessibility or quantify the electrostatic component of MTS modification. MTSES<sup>–</sup> and MTSET<sup>+</sup> are comparable in size to arginine

and were therefore used in *Paper IV* to test the importance of gating charges for PUFA sensitivity.



**Figure 9.** Principle of MTSEA<sup>+</sup> modification to localize the PUFA-action site. **A)** The PUFA has an apparent pKa of 7.4. **B)** Insertion of MTSEA<sup>+</sup> close to the PUFA deprotonates the carboxyl charge making the PUFA more potent. **C)** Insertion of MTSEA<sup>+</sup> distant from the PUFA will not change the degree of protonation.

### 3.5. Screening of surface charges with metal ions

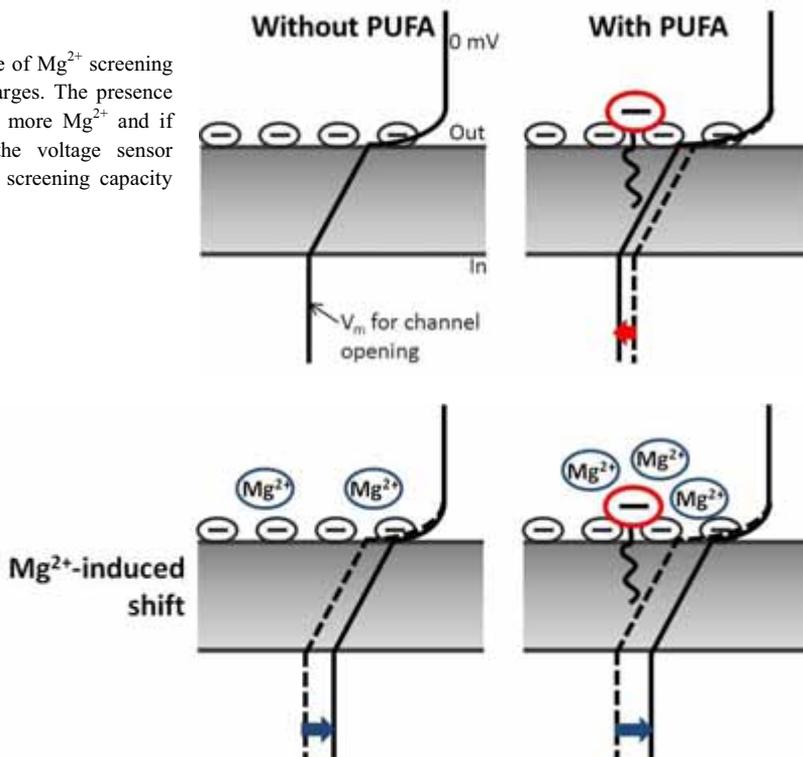
That metal ions shift the voltage dependence of voltage-gated ion channels was shown more than 50 years ago by Bernhard Frankenhaeuser and Alan Hodgkin (Frankenhaeuser & Hodgkin, 1957). Mechanisms underlying metal ion effects have been extensively reviewed (Hille, 2001; Elinder & Århem, 2003). It is for instance known that the  $G(V)$  curve of the Shaker channel is shifted to positive voltages by extracellular application of Mg<sup>2+</sup> (Figure 10, left panel) (Elinder et al., 1998). This is because Mg<sup>2+</sup> screens fixed negative charges on the surface of the membrane and channel, altering the electric field sensed by the channel. Higher membrane voltages are therefore needed to open the channel. The screening capacity of Mg<sup>2+</sup> can be used to elucidate if a fixed charge (e.g. a PUFA) is located close to the voltage sensor. Such experiments were performed in *Paper II*. A PUFA carboxyl charge close to S4 left-shifts the channel voltage dependence (Figure 10, upper panel) but also attracts more Mg<sup>2+</sup>. This is seen as an increased screening capacity of Mg<sup>2+</sup> (measured as a larger Mg<sup>2+</sup>-induced  $G(V)$  shift) if the PUFA is close to the voltage sensor (Figure 10, lower panel).

The theoretical screening capacity of Mg<sup>2+</sup> is expressed by the Grahame equation describing how the surface charge density ( $\sigma$ ) is related to the surface potential ( $\psi$ ):

$$\sigma^2 = 2 \varepsilon_r \varepsilon_0 R T \sum c_i [\exp(-z_i F \psi R^{-1} T^{-1}) - 1], \quad (\text{Eq. 4})$$

where  $R$ ,  $T$ , and  $F$  have their normal thermodynamic meanings,  $\varepsilon_r$  is the dielectric constant of the medium ( $\sim 80$  for the extracellular solution),  $\varepsilon_0$  the permittivity of free space, and  $c_i$  the bulk concentration and  $z_i$  the valence of the  $i$ th ionic species in the solution.

**Figure 10.** Principle of  $Mg^{2+}$  screening of fixed surface charges. The presence of a PUFA attracts more  $Mg^{2+}$  and if located close to the voltage sensor results in increased screening capacity of  $Mg^{2+}$ .



### 3.6. Theoretical calculations of PUFA effects

Theoretical calculations were used in *Papers II* and *IV* to help understand and quantify experimental data. First, a simple four-state model of the pH dependence of free fatty acid effects was developed in *Paper II*. Experimental DHA data at different pH can be explained if both the PUFA and the PUFA-action site on the channel is pH dependent. Higher pH increases the PUFA potency (through deprotonation) and the affinity of the PUFA – channel interaction. The model is expressed mathematically as:

$$\Delta V = \Delta V_{\max} / ((1 + 10^{(pK_{a,FFA} - pH)}) \times (1 + K_D/[L] \times (1 + 10^{(pK_{a,rec} - pH)}))), \quad (\text{Eq. 5})$$

where  $\Delta V$  is the fatty acid-induced  $G(V)$  shift,  $\Delta V_{\max}$  the maximum possible induced shift,  $pK_{a,rec}$  and  $pK_{a,FFA}$  the  $pK_a$  for the action site and free fatty acid respectively.  $K_D$  is the lowest possible combined dissociation constant for the interaction between fatty acid and channel, and  $[L]$  the fatty acid concentration.

In *Paper IV*, a modified Coulomb's law (McLaughlin, 1989; Elinder & Århem, 2003) was used to calculate the distance between the PUFA carboxyl charge and the gating charges in S4 based on DHA-induced  $Q(V)$  and  $G(V)$  shifts.

If a charge is located at the border between a low dielectric medium (the lipid membrane) and a high dielectric medium (water), the potential  $\psi$  at the distance  $r$  from an elementary charge  $e_0$  (e.g. the PUFA charge) can be calculated as:

$$\psi(r) = 2 e_0 \exp(-\kappa r)/(4 \pi \varepsilon_0 \varepsilon_a r), \quad (\text{Eq. 6})$$

where  $\varepsilon_0$  is the permittivity of free space ( $8.85 \times 10^{-12} \text{ Fm}^{-1}$ ),  $\varepsilon_a$  is the relative dielectric constant of the aqueous phase (80), and  $\kappa$  is the inverse of the Debye length in the aqueous phase (9.8 Å in the 1K solution, see e.g. (Elinder et al., 2001a)). Despite the very simplistic calculations, Eq. 6 has previously been proven useful in localizing Shaker channel S4 (Elinder et al., 2001b; Broomand & Elinder, 2008).

### 3.7. Computer simulations

The importance of measured PUFA effects on cellular excitability is difficult to assess experimentally because PUFAs also affect other ion channels (see Discussion). In *Paper I* and *III* we therefore performed computer simulations of PUFA effects on excitability using a model of the node of Ranvier of the frog myelinated axon (Frankenhaeuser & Huxley, 1964). This system is based on a  $\text{Na}_v$  channel, a delayed-rectifier  $\text{K}_v$  channel, and a leakage channel. Repetitive firing, defined as firing after more than 100 ms of continuous stimulation, was induced by persistent stimulus currents. The impact of small changes in channel voltage dependence on repetitive firing was tested by shifting the voltage dependence of the delayed-rectifier  $\text{K}_v$  channel (*Paper I* and *III*) or  $\text{Na}_v$  channel (*Paper III*) in either direction along the voltage axis.

In *Paper IV* we also performed kinetic modelling on the simple six-state gating model (Figure 3B) to evaluate experimental DHA data from gating current and ion current measurements. In the model, transitions between different states depend on forward and backward rate constants. To model PUFA effects on early and final S4 transitions respectively, the voltage at which the forward and backward transitions are equal was shifted according to the PUFA-induced shift of the  $G(V)$  and  $Q(V)$  curves of the ILT mutant.

### 3.8. Clinical data

Patient data and cerebrospinal fluid samples in *Paper I* were collected by Maria Dahlin and Per Åmark at the Astrid Lindgren Children's Hospital. The study was approved by the local Ethics Committee at the Karolinska Hospital and informed consent obtained from patients and/or parents. All patients were classified with intractable epilepsy with a prior trial of at least three antiepileptic drugs. Cerebrospinal fluid was collected by lumbar puncture before starting ketogenic diet treatment and 3-6 month after starting ketogenic diet treatment. Patient demographic data, procedure for ketogenic diet treatment and details for cerebrospinal fluid collection are described in *Paper I*.

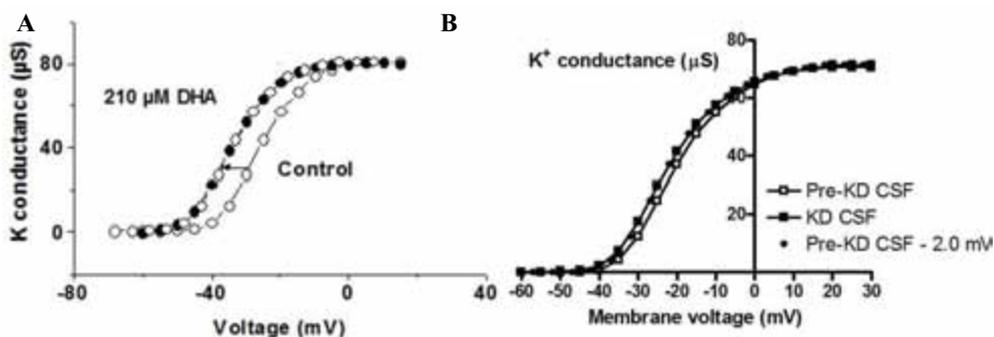


## 4. RESULTS

Below I will shortly describe the main findings of the papers (see each paper for further details) and then elaborate on the importance in the Discussion.

### 4.1. PUFAs and cerebrospinal fluid from children on the ketogenic diet shift Shaker channel voltage dependence to more negative voltages

*Paper I* was our first study of possible PUFA and ketone body effects on the Shaker channel. We found that all three tested PUFAs (DHA, EPA, and linoleic acid) enhanced channel activity by shifting the  $G(V)$  curve in negative direction along the voltage axis (Figure 11A). This means that the  $K_v$  channel opens at more negative voltages, in line with what we expect from a substance with antiepileptic effects. The shift was dose dependent saturating at  $-9.6$  mV for DHA at pH 7.4, and  $K_D$  was determined to  $79 \mu\text{M}$  (Eq. 3). The DHA-induced shift was also significant for clinically relevant concentrations.  $21 \mu\text{M}$  DHA, estimated to reflect the ketogenic diet-induced rise in cerebrospinal fluid PUFAs, robustly shifted the  $G(V)$  curve with  $-2.3$  mV. In contrast, the two tested ketone bodies (DL- $\beta$ -hydroxybutyric acid and acetoacetic acid) did not affect the Shaker channel and were therefore not studied further. We also tested if cerebrospinal fluid from 20 children on the ketogenic diet mimicked the effect of pure PUFAs, which would support that PUFAs in cerebrospinal fluid protect against epileptic seizures. Indeed, ketogenic diet treatment in our patient group reduced seizure frequency, and cerebrospinal fluid collected during ketogenic diet treatment significantly shifted the  $G(V)$  curve of the Shaker channel compared to cerebrospinal fluid collected before treatment (Figure 11B). The mean shift for all 20 patients was  $-0.6$  mV with individual shifts up to  $-2.8$  mV. The mean shift corresponds approximately to a  $5.1 \mu\text{M}$  increase in cerebrospinal fluid PUFAs (Eq. 3) which is lower than our predicted  $21 \mu\text{M}$ . One reason for this discrepancy could be that PUFAs in cerebrospinal fluid are bound to transport proteins.



**Figure 11.** The effect of DHA and cerebrospinal fluid on the Shaker channel. **A)** DHA shifts the voltage dependence to negative voltages. **B)** Cerebrospinal fluid (CSF) from children on the ketogenic diet (KD) induces a negative shift of the voltage dependence compared to cerebrospinal fluid collected before ketogenic diet treatment. This particular cerebrospinal fluid sample induced a  $-2.0$  mV shift. (Figures adapted from *Paper I*)

## 4.2. A *cis*-polyunsaturated acyl tail is critical for the PUFA effect

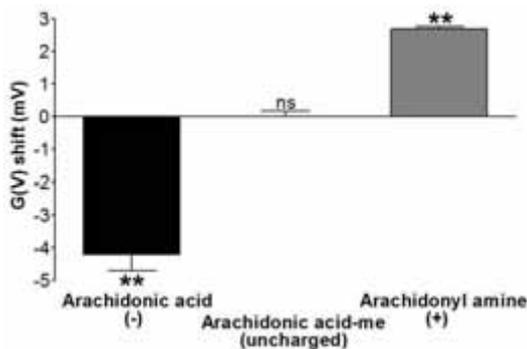
To understand how the three tested PUFAs in *Paper I* induce channel opening we aimed at determining which structural properties are needed for the effect. There is a general discussion about possible beneficial and harmful effects of unsaturated and saturated fatty acids, respectively, and we therefore started to elaborate on the role of the lipophilic acyl tail. Table II shows the classification of all tested fatty acids in *Paper II*. Interestingly, all PUFAs showed similar potency to change the voltage dependence of the Shaker channel, while monounsaturated fatty acids (one double bond) and saturated fatty acids (no double bond) did not induce negative shifts. Furthermore, the double bonds needed to be in the flexible *cis*-configuration. When substituting the *cis*-double bonds in the PUFA linoleic acid with *trans*-double bonds the effect on the voltage dependence was completely abolished.

## 4.3. The charge of the PUFA determines the direction of the shift

The other structural component of a free fatty acid is the carboxyl head group which is negatively charged when deprotonated and uncharged when protonated. In *Paper II* and *III* we demonstrated the critical role of the carboxyl charge by three different approaches:

- 1) The PUFA effect was highly pH dependent and followed a pattern predictable from the state of protonation: Low pH protonates the PUFA and abolished the effect while the PUFA potency increased dramatically with increasing extracellular pH. Also, a Shaker channel mutant with three additional positive charges (Broomand et al., 2007) on the surface (corresponding to 0.3 units higher local pH) was more sensitive to PUFAs. The apparent pKa for DHA in our experimental setting was derived to 7.4 in *Paper II* by putting experimental DHA data into the model described by Eq. 5 and solving it with global least-square fit.
- 2) PUFAs increased the screening capacity of  $Mg^{2+}$  supporting a position of the carboxyl charge close to the voltage sensor (see Figure 9 in Methodology).
- 3) The most important evidence for a crucial role of the carboxyl charge came from charge-substituting experiments. When esterifying the carboxyl group to an uncharged methyl group (PUFA-me) in *Paper II*, the PUFA effect was completely abolished. In *Paper III* we tested the potency of three substances with identical polyunsaturated acyl tails (corresponding to arachidonic acid) but with different charge. As expected from an electrostatic mechanism, the  $G(V)$  curve shifted in negative direction by negatively charged arachidonic acid and in positive direction by positively charged arachidonyl amine. Again, uncharged arachidonic acid methyl ester did not change the voltage dependence. Thus, lipophilic substances shift the voltage dependence in either direction along the voltage axis depending on their charge (Figure 12).

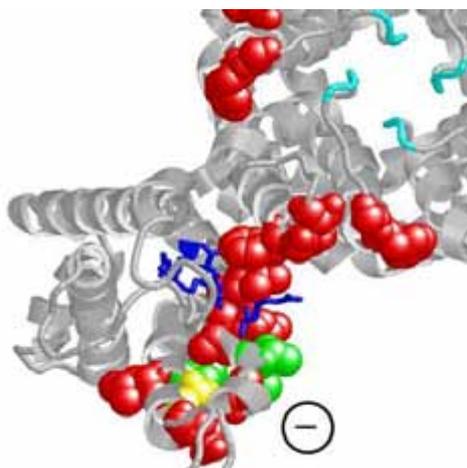
These experiments together demonstrate that the negative PUFA carboxyl charge is critical to induce Shaker channel opening and suggest an electrostatic interaction between the PUFA and the voltage sensor.



**Figure 12.** The effect of differently charged substances on the voltage dependence of the Shaker channel. (Figure adapted from *Paper III*)

#### 4.4. PUFAs act on a lipid-exposed surface on the voltage-sensor domain

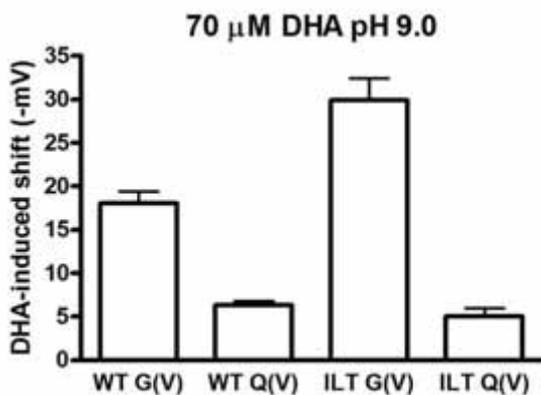
In *Paper II* we suggested that PUFAs induce channel opening by a novel pharmacological mechanism which we called the lipoelectric mechanism. In the lipoelectric mechanism, PUFAs induce channel opening by incorporating into a lipophilic site and electrostatically attracting the voltage sensors. A facilitation of the outward S4 movement shifts the  $G(V)$  curve in negative direction. PUFAs need to localize relatively close to S4 to affect voltage-sensor movement electrostatically. Their lipophilicity also requires a hydrophobic environment for incorporation. In *Paper IV* we used a systematic mutagenesis approach to identify the PUFA-action site. 17 residues distributed over helices S3-S6 fulfilling these criteria were selected for point-cysteine mutagenesis followed by charge modification with MTSEA<sup>+</sup> to determine which part of the channel is important for PUFA efficiency (see Methodology for details). We found that critical residues were restricted to the extracellular ends of S3 and S4, facing the surrounding phospholipids (residues with largest impact in green, smaller impact in yellow, and no impact in red in Figure 13). None of the residues in the pore domain were important for PUFA efficiency. This suggests that the PUFAs incorporate into the lipid bilayer adjacent to S3 and S4, but distant to the pore domain.



**Figure 13.** Suggested PUFA-action site on the Shaker channel. Top view of the K<sub>v</sub>1.2/2.1 chimera with Shaker side chains. Yellow (T329) and green (I325, A359, and I360) residues are important for PUFA sensitivity as tested by cysteine mutagenesis and MTSEA<sup>+</sup> modification. Red residues have no impact on PUFA sensitivity. Gating charges in S4 coloured blue and the selectivity filter in cyan. The negative charge indicates the approximate position of the PUFA carboxyl charge. (Figure adapted from *Paper IV*)

#### 4.5. PUFAs mainly act on the final voltage-sensor transition

The localization of the PUFA-action site and lack of critical residues in the pore domain support the hypothesis that PUFAs act directly on the voltage sensor. To further understand how PUFAs induce channel opening by voltage sensor interactions, we investigated which transition during channel activation the PUFAs exert their primary effect on. In *Paper II* we measured gating currents from the Shaker/W434F channel that mainly reports on early voltage-sensor movements between different closed conformations. We would expect a large PUFA effect on the gating current if PUFAs localize close to S4, facilitating the outward S4 movement by acting as fixed negative surface charges. The DHA effect on Shaker channel gating currents ( $Q(V)$  in Figure 14) was however only about 1/3 of the effect on the  $G(V)$  curve, suggesting that PUFAs primarily act on the final voltage-sensor transition. This was an unexpected experimental finding, as the final voltage-sensor movement is suggested to be mainly in the plane of the membrane, meaning that PUFAs need to facilitate some kind of horizontal movement. Isolated effects on early S4 movements and the final voltage-sensor transition are difficult to study in the Shaker channel because of their overlapping voltage intervals (see Methodology). In *Paper IV* we therefore repeated the measurements on the Shaker ILT mutant with clearly separated early and final S4 movements. The more accurate quantification together with kinetic modelling verified that PUFAs have a minor effect on the early voltage-sensor transitions but mainly act on the final voltage-sensor transition (with a ratio of about 1:6, Figure 14).

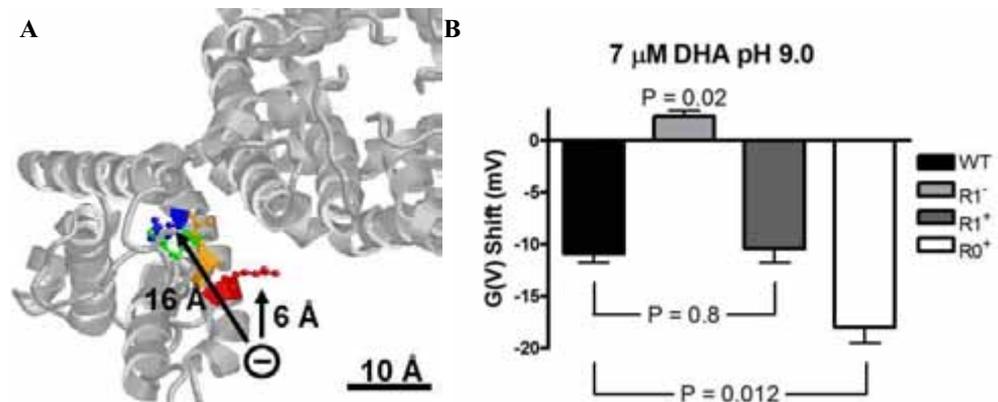


**Figure 14.** DHA effects on early ( $Q(V)$ ) and final ( $G(V)$ ) voltage-sensor transition measured in WT and ILT-mutated channels. (Modified figure from *Paper IV*)

#### 4.6. The profile of the outermost gating charges is critical for the PUFA effect

How can PUFAs electrostatically affect the final voltage-sensor transition? In *Paper IV*, PUFA-induced shifts of early and final voltage-sensor movements were translated to distances between S4 gating charges and the PUFA carboxyl charge using Eq. 6. If the PUFAs act purely electrostatically, the effect on the early transitions corresponds to a distance between PUFA and S4 of 15 Å while the effect on the final voltage-sensor movement corresponds to only 6 Å. This suggests that a gating charge of Shaker moves  $\sim 9$  Å closer to the PUFA during the final S4 movement. These relatively blunt calculations (but previously proven accurate

(Elinder et al., 2001b; Broomand & Elinder, 2008)) agree remarkably well with the position of the gating charges in the open  $K_v$  crystal structure and the suggested PUFA localization in the lipid bilayer (Figure 15A). In the structure, the deduced PUFA carboxyl charge is about 16 Å away from the position where the gating charges emerge on the channel surface, and about 6 Å away from the final position of the outermost gating charge (R1) in its most up state. To experimentally test the hypothesis that PUFAs electrostatically facilitate this final ‘swinging’ S4 movement by attracting R1, we changed the charge of R1 from positive to negative by mutating it to a cysteine then modified with MTSES<sup>-</sup>. The R1 charge mutation completely abolished the ability of DHA to facilitate channel opening and even induced a small but significant positive shift of the  $G(V)$  curve (R1<sup>-</sup> in Figure 15B). PUFA sensitivity was rescued if R1 instead was modified with MTSET<sup>+</sup> (R1<sup>+</sup> in Figure 15B). Thus, R1 is important for the PUFA effect and the charge of the residue seems to be the critical factor. The crystal structure also suggests that position R0, three residues extracellular to R1, could interact with the PUFA by pointing in the same direction as R1. When adding a positive charge at the originally uncharged R0 position, the Shaker channel got almost twice as sensitive to DHA (R0<sup>+</sup> in Figure 15B). Altogether, these experiments demonstrate the critical role of the outermost gating charges for PUFA potency.

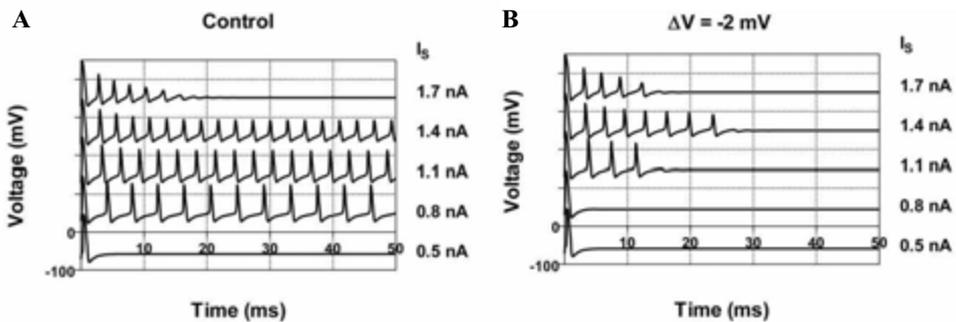


**Figure 15.** The outermost gating charges are critical for PUFA sensitivity. **A)** Distance between suggested PUFA location and gating charges in the  $K_v1.2/2.1$  crystal structure with Shaker side chains. R1-R4 shown as sticks. **B)** The charge at positions R0 and R1 is important for the PUFA sensitivity as seen by MTSES<sup>-</sup> or MTSET<sup>+</sup> modification. Experiments performed in high pH to push DHA into a deprotonated state avoiding interference from changes in local pH. (Figures adapted from *Paper IV*)

#### 4.7. Small $G(V)$ shifts prevent repetitive firing in computer simulations

Most experiments in *Papers I-IV* aiming at unravelling the PUFA mechanism were done with relatively high PUFA concentrations or in high pH to induce large effects for robust quantification. However, we have also shown that clinically relevant PUFA concentrations induce significant  $G(V)$  shifts (-2.3 mV on Shaker WT in *Paper I* and -4.4 mV for the triple-positive mutant in *Paper II*). Although statistically significant, could these seemingly small shifts impact an epileptic event? To evaluate this in a setting without interference from PUFA

effects on other channels we performed computer simulations on the myelinated frog nerve fibre which is a simple but well-known excitable system. We showed in *Paper I* that epileptic activity generated by stimulating currents of different size was effectively abolished when shifting the voltage dependence of  $K_v$  channels with only  $-2$  mV (Figure 16). The relationship between  $K_v$  channel  $G(V)$  shifts and the corresponding change in number of  $K_v$  channels was found to be satisfactorily described with Eq. 1 using a slope factor  $s$  of  $4.7$  mV (*Paper III*). Thus, the  $-2$  mV shift is functionally equivalent to increasing the number of  $K_v$  channels with 53%. The mean cerebrospinal fluid-induced shift of  $-0.6$  mV is equivalent to increasing the number of  $K_v$  channels with 14%.



**Figure 16.** Small shifts of  $K_v$  channel voltage dependence abolish repetitive firing in computer simulations on the myelinated frog axon. **A)** Stimulating currents ( $I_s$ ) induce repetitive firing. **B)** Repetitive firing is effectively abolished by shifting the voltage dependence of a delayed-rectifier  $K_v$  channel with  $-2$  mV. (Figures adapted from *Paper I*)

We further elaborated on the impact of small  $K_v$  channel shifts in a review paper (Börjesson & Elinder, 2008) showing that the protective effect is universal and not restricted to specific  $Na_v$  and  $K_v$  channel densities. In *Paper III* we expanded the computer simulations to include effects of positive  $K_v$  channel shifts and shifts in either direction for  $Na_v$  channel activation and inactivation. Isolated shifts of only a few mV in each and one of these parameters induced or prevented repetitive firing, again suggesting that small changes in the voltage dependence could have dramatic effects on neuronal excitability.

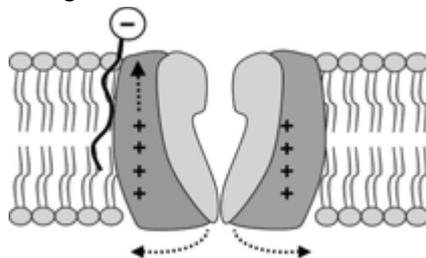
## 5. DISCUSSION

In this thesis work, the effect of PUFAs on the Shaker  $K_v$  channel expressed in *Xenopus* oocytes was studied with the two-electrode voltage clamp technique. Olaf Andersen and colleagues have shown that PUFAs indirectly modulate the activity of membrane proteins, including voltage-gated ion channels, by changing the properties of the membrane (Andersen & Koeppe, 2007). In this thesis we instead propose a direct PUFA effect on  $K_v$  channels, which we call lipoelectric modulation. In the following sections, I will elaborate on the molecular mechanism of lipoelectric modulation, the possible role of PUFAs in the ketogenic diet, and lipoelectric modulation as future pharmacological strategy.

### 5.1. Lipoelectric modulation of $K_v$ channel activity

From experiments supporting an electrostatic mechanism of action we initially believed that PUFAs simply facilitate the outward S4 movement by providing additional fixed negative surface charges close to the voltage sensor. A schematic figure for this mechanism was

presented in *Paper II* and a review paper (Börjesson & Elinder, 2008) and is shown in Figure 17. This is the mechanism by which extracellular channel glycosylation (Watanabe et al., 2007; Johnson & Bennett, 2008) and some cationic metal ions are suggested to alter channel activity (Elinder & Århem, 2003). However, gating current measurements clearly showed that the early voltage-sensor movements are only slightly affected by PUFAs while the final voltage-sensor transition is the main target. This surprised us but was also inspiring because the nature of the final movement was not known.



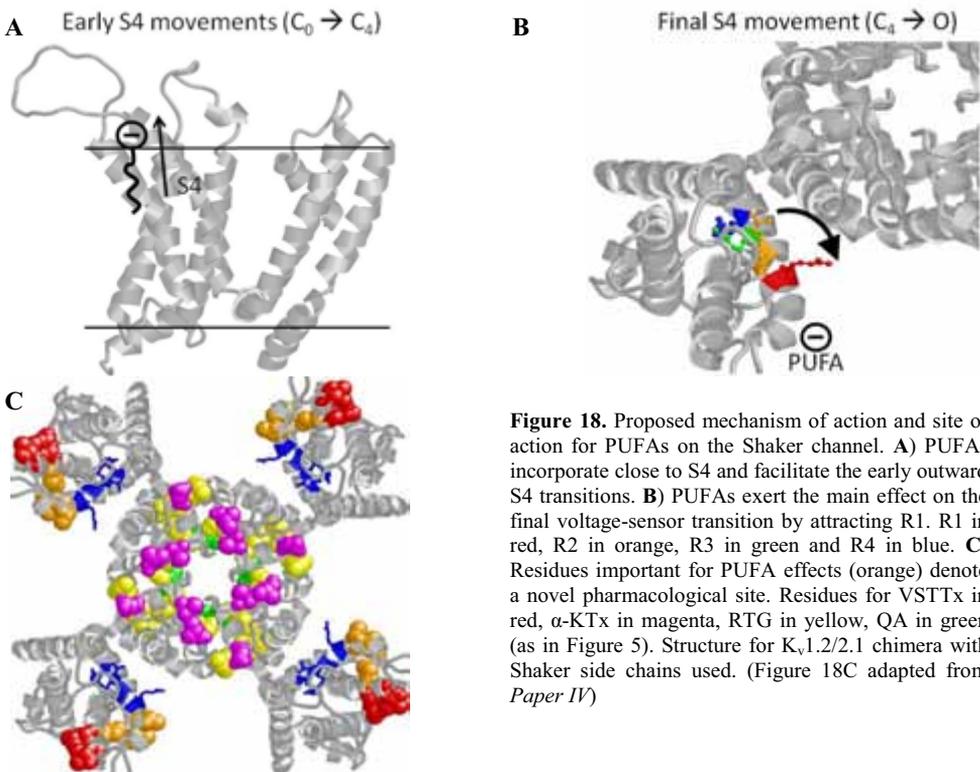
**Figure 17.** Schematic illustration of the initially suggested lipoelectric mechanism. (Figure adapted from Börjesson & Elinder, 2008)

Also, there was to our knowledge no substance reported to specifically target this movement. PUFAs could therefore act as model substances unravelling a novel pharmacological mechanism of action.

#### 5.1.1. Proposed model for PUFA-induced $K_v$ channel opening

Figure 18 shows our current model for how we imagine PUFA-induced opening of the Shaker channel. PUFAs bind to the lipid bilayer between two channel subunits, adjacent to helices S3 and S4. The negative carboxyl charge has a small effect on the early, outward S4 movement by acting as a surface charge (Figure 18A). This is followed by the larger effect on the final voltage-sensor transition (Figure 18B). The nature of this movement is still not clear but believed to be primarily in the plane of the membrane generating only a small gating current (Pathak et al., 2005). If speculating that the final movement is the last rotational step in the helical-screw model of channel opening, the outermost gating charge R1 moves from a position  $\sim 16$  Å away from the PUFA to only  $\sim 6$  Å away (Figure 18B). This agrees well with our experimental data and electrostatic calculations suggesting that PUFAs primarily act by attracting R1 which facilitates the final ‘swing’. As discussed in *Paper IV*, our data is, besides

the helical-screw model, also consistent with other models suggesting a horizontal S4 movement during the final transition. At this point we cannot distinguish between these models. However, a recent study suggesting also a transmembrane S4 movement during the final opening step (Phillips & Swartz, 2010) supports the helical-screw model. Both the PUFA mechanism of action and the action site are novel. Figure 18C shows that residues important for PUFA potency (orange) are close to, but distinct from, the site for voltage-sensor trapping toxins (red). The PUFA-action site is also distinct from the proposed sites for two recently described  $K_v7$  channel openers, NH29 (Peretz et al., 2010) and ICA-27243 (Padilla et al., 2009), targeting the central part of the voltage-sensor domain.

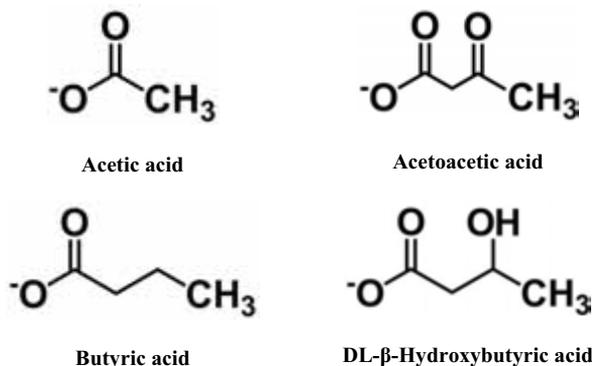


**Figure 18.** Proposed mechanism of action and site of action for PUFAs on the Shaker channel. **A)** PUFAs incorporate close to S4 and facilitate the early outward S4 transitions. **B)** PUFAs exert the main effect on the final voltage-sensor transition by attracting R1. R1 in red, R2 in orange, R3 in green and R4 in blue. **C)** Residues important for PUFA effects (orange) denote a novel pharmacological site. Residues for VSTTx in red,  $\alpha$ -KTx in magenta, RTG in yellow, QA in green (as in Figure 5). Structure for  $K_v1.2/2.1$  chimera with Shaker side chains used. (Figure 18C adapted from *Paper IV*)

### 5.1.2. Structural fatty acid components needed for the effect

The negative carboxyl charge in combination with a *cis*-polyunsaturated acyl tail was identified to be the critical structural components of free fatty acids to induce channel opening. The importance of the charge is obvious for an electrostatic mechanism. To us, however, the need of double bonds is less intuitive. The introduction of *cis*-double bonds in an acyl tail increases the flexibility (Feller et al., 2002), which may be of importance in the lipoelectric mechanism. It is a clear break-point in the effect between fatty acids with one and two double bonds and no seemingly potentiation by additional double bonds. Also, there is no difference between  $\omega$ -3 and  $\omega$ -6 PUFAs suggesting that the exact position of the double bonds is not important. One speculative explanation would be that two *cis*-double bonds provide the

critical flexibility for positioning the carboxyl charge close to S4, and that additional flexibility cannot further optimize the position. In light of *Paper II*, it is not surprising that the ketone bodies acetoacetic acid and DL- $\beta$ -hydroxybutyric acid fail to induce Shaker channel  $G(V)$  shifts. Both ketone bodies have short saturated acyl chains sharing structural similarities with acetic acid and butyric acid (Figure 19), respectively, shown to be ineffective in *Paper II*.



**Figure 19.** Comparison of chemical structures of the fatty acids acetic acid and butyric acid and the ketone bodies acetoacetic acid and DL- $\beta$ -hydroxybutyric acid.

### 5.1.3. Channel properties deciding PUFA sensitivity

Experiments in this thesis are restricted to the Shaker channel, but there are also other  $K_v$  channels which are sensitive to PUFAs (Boland & Drzewiecki, 2008), some possibly via lipoelectric modulation. This is supported by experiments on  $K_v7$  channels where PUFAs induce negative  $G(V)$  shifts (Börjesson et al., unpublished data). PUFAs can also induce  $K_v$  channel inhibition (Boland & Drzewiecki, 2008), normally measured as current reduction at positive voltages, by mechanisms distinct from lipoelectric modulation (Decher et al., 2010). High PUFA concentrations induce channel inhibition also in our experiments (*Paper II*). It is therefore possible that lipoelectric  $G(V)$  shifts of other  $K_v$  channels are unmasked if lower PUFA concentrations were used. Furthermore, despite PUFA-induced current reduction at positive voltages, the  $K_v1.5$  channel shows an absolute increase in  $K^+$  current at negative voltages (Honore et al., 1994), and additional  $K_v$  channels display negative  $G(V)$  shifts (e.g. Poling et al., 1996; McKay & Jennings, 2001; Guizy et al., 2008).

The crucial role of the charges at positions R0 and R1 for PUFA sensitivity opens up for the possibility that different channels may be differently sensitive to lipoelectric substances depending on their gating-charge profile. Table III shows a sequence alignment of the S4 segment of the Shaker channel and two other  $K_v$  channels with different gating charge distributions.  $K_v4.1$ , lacking gating charges at both position R0 and R1, could theoretically be less sensitive to PUFAs.  $K_v2.1$  has two arginines in the extracellular end of S4 (however lacking R1), potentially interacting effectively with PUFAs. The gating charge distribution of different channels could be exploited when designing substances with preferential effects on certain channels, but also for understanding which channels are targeted by PUFAs.

**Table III.** Example of  $K_v$  channels with different gating charge profile. Positive gating charges in bold and underlined.  $\alpha$ -helical S4 segment indicated above the sequences.

	R0	R1	R2	R3	R4	K5	R6	K7
<b>Shaker</b>	A M S L A I L <u><b>R</b></u> V I <u><b>R</b></u> L V <u><b>R</b></u> V F <u><b>R</b></u> I F <u><b>K</b></u> L S <u><b>R</b></u> H S <u><b>K</b></u>							
<b><math>K_v4.1</math></b>	V S G A F V T L <u><b>R</b></u> V F <u><b>R</b></u> V F <u><b>R</b></u> I F <u><b>K</b></u> F S <u><b>R</b></u> H S Q							
<b><math>K_v2.1</math></b>	Q N V <u><b>R</b></u> <u><b>R</b></u> V V Q I F <u><b>R</b></u> I M <u><b>R</b></u> I L <u><b>R</b></u> I L <u><b>K</b></u> L A <u><b>R</b></u> H S T							

## 5.2. PUFA modulation of voltage-gated ion channel activity as mechanism of the ketogenic diet?

Numerous studies have tried to unravel the mechanism of the ketogenic diet and one reason is the desire to, as Rho and Sankar phrase it, “pack the ketogenic diet into a pill” (Rho & Sankar, 2008). One popular hypothesis has been a ketogenic diet-induced shift towards production of the inhibitory neurotransmitter GABA, which is indeed elevated following ketogenic diet treatment (reviewed in Bough & Rho, 2007). Also the 20-fold increase in ketone bodies has been subject to extensive studies, suggesting an antiexcitable effect as  $K_{ATP}$  channel opens via a reduction of ATP (Ma et al., 2007; Yellen, 2008). However, as described in the Introduction, PUFAs are also considered as top-candidates for the antiepileptic effect of the ketogenic diet.

### 5.2.1. Role of PUFA-induced $K_v$ channel opening?

One aim of this thesis was to test if PUFA-induced  $K_v$  channel opening could contribute to the antiexcitable effect of the ketogenic diet. The impact of  $K_v$  channel effects on neuronal excitability is difficult, if not impossible, to test experimentally because PUFAs also act on other neuronal channels (see below). We therefore performed computer simulations to evaluate the isolated effect on  $K_v$  channels. As mentioned in the Results section,  $K_v$  channel shifts induced by clinical PUFA concentrations effectively prevent repetitive firing in simulations on a simple excitable membrane with a delayed-rectifier K channel. Analogous simulations on the considerable more complex CA1 pyramidal neuron system also suggest that small shifts of A-type channel activation effectively prevent epileptic activity (Tigerholm, Lundberg, Fransén, Royal Institute of Technology, Stockholm, Sweden, unpublished data). The smaller than expected effect of cerebrospinal fluid from children on the ketogenic diet may result from low concentrations of effective free PUFAs in cerebrospinal fluid. PUFAs circulate mainly bound to albumin or incorporated into lipoproteins (van der Vusse et al., 2000; Hamilton & Brunaldi, 2007), and are in vivo either dissociated from albumin or released from lipoproteins by lipases for effective membrane incorporation. These mechanisms may not work identically in the oocyte system. It is also known that considerable higher substance concentrations may be needed on *Xenopus* oocytes to generate comparable channel effects to mammalian expression systems (Rolf et al., 2000).

### 5.2.2 PUFAs inhibit $Na_v$ and $Ca_v$ channels

We believe PUFA opening of  $K_v$  channels to be one important component in the ketogenic diet working together with other mechanisms to achieve seizure control. One collaborating mechanism could be PUFA modulation of other ion channel (Boland & Drzewiecki, 2008).

For instance, PUFAs can shift  $\text{Na}_v$  and  $\text{Ca}_v$  channel activation to more negative voltages (which increases excitability) but more importantly always shift inactivation even more (Hong et al., 2004; Danthi et al., 2005; reviewed in Leaf et al., 2005). Thus, the net effect is  $\text{Na}_v$  and  $\text{Ca}_v$  channel inhibition. Our computer simulations show that small shifts in the voltage dependence of activation or inactivation of  $\text{Na}_v$  channels profoundly affect excitability. The impact of minor changes in  $\text{Na}_v$  channel activity is also shown by epilepsy-inducing mutations shifting channel activation to more negative voltages by only  $\sim 5$  mV (Xu et al., 2007), and computer simulations mimicking these effects (Thomas et al., 2007; Thomas et al., 2009). Why PUFAs have dominating effects on inactivation is not known and it is not clear how PUFAs interact with  $\text{Na}_v$  and  $\text{Ca}_v$  channels. The carboxyl charge is established to be crucial for the PUFA effect on cardiac  $\text{Na}_v$  channels (Kang & Leaf, 1996; Bendahhou et al., 1997). It is therefore possible that lipoelectric modulation is the mechanism of action. It is also tantalizing to speculate on a larger PUFA effect on the voltage sensor of domain IV (mainly responsible for inactivation) than on the voltage sensors of domain I-III (involved in activation) (Chen et al., 1996; Keynes & Elinder, 1998).

### **5.3. Lipoelectric modulation as future pharmacological strategy?**

The PUFAs have served as excellent test substances to identify a novel pharmacological mechanism. They do, however, have shortcomings as medical drugs by being very promiscuous, acting on many targets although with different potency. We believe lipoelectric modulation to be an interesting future pharmacological strategy if compounds with higher specificity are designed to minimize adverse effects. This could be achieved if substances interact with or bind to certain lipid-exposed (or hydrophobic pocket-exposed) residues close to S4 with high affinity. In a related project we aim at identifying lipoelectric substances with higher selectivity (Ottosson et al, unpublished data).

Obvious conditions suitable for lipoelectric modulation are epilepsy, heart arrhythmias, and pain, where opening of  $\text{K}_v$  channels could reduce excitability in each system. The beauty of electrostatic interactions is that opposite effects are accomplished if the charge is switched. Positively charged lipoelectric substances could therefore dampen excitability by reducing  $\text{Na}_v$  and  $\text{Ca}_v$  channel activity, or increase neuronal excitability during for instance multiple sclerosis by targeting  $\text{K}_v$  channels.



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