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Polyunsaturated fatty acids are potent openers of human M-channels expressed in Xenopus laevis oocytes

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Short title: Fatty acids open the M-channel

Abstract

Aim: Polyunsaturated fatty acids have been reported to reduce neuronal excitability, in part by promoting inactivation of voltage-gated sodium and calcium channels. Effects on neuronal potassium channels are less explored and experimental data ambiguous. The aim of this study was to investigate anti-excitability effects of polyunsaturated fatty acids on the neuronal M-channel, important for setting the resting membrane potential in hippocampal and dorsal root ganglion neurons.

Methods: Effects of fatty acids and fatty-acid analogues on mouse dorsal root ganglion neurons and on the human K_v7.2/3 channel expressed in *Xenopus laevis* oocytes were studied using electrophysiology.

Results: Extracellular application of physiologically relevant concentrations of the polyunsaturated fatty acid docosahexaenoic acid hyperpolarized the resting membrane potential (-2.4 mV by 30 μ M) and increased the threshold current to evoke action potentials in dorsal root ganglion neurons. The polyunsaturated fatty acids docosahexaenoic acid, α -linolenic acid, and eicosapentaenoic acid facilitated opening of the human M-channel, comprised of the heteromeric human K_v7.2/3 channel expressed in *Xenopus* oocytes, by shifting the conductance-*versus*-voltage curve towards more negative voltages (by -7.4 to -11.3 mV by 70 μ M). Uncharged docosahexaenoic acid methyl ester and monounsaturated oleic acid did not facilitate opening of the human K_v7.2/3 channel.

Conclusions: These findings suggest that circulating polyunsaturated fatty acids, with a minimum requirement of multiple double bonds and a charged carboxyl group, dampen excitability by opening neuronal M-channels. Collectively, our data bring light to the molecular targets of polyunsaturated fatty acids and thus a possible mechanism by which polyunsaturated fatty acids reduce neuronal excitability.

Keywords: electrophysiology, epilepsy, excitability, KCNQ, M-channel, polyunsaturated fatty acids

Introduction

Polyunsaturated fatty acids (PUFAs) have been reported to reduce neuronal excitability (Taha *et al.* 2010a). PUFAs increase seizure thresholds (Voskuyl *et al.* 1998; Xiao & Li 1999; Taha *et al.* 2010b; Bandero *et al.* 2013; Taha *et al.* 2013) and pain thresholds (Yehuda *et al.* 1993; Nakamoto *et al.* 2010) in rats and mice, and reduce neuronal excitability *in vitro* (Xiao & Li 1999; Young *et al.* 2000). These effects of PUFAs in cellular and animal studies indicate that PUFAs may protect against hyperexcitability in epilepsy and pain. We and others have suggested that PUFAs contribute to the dampening effect of the fat-rich ketogenic diet (Cunnane *et al.* 2002; Xu *et al.* 2008), in which the serum concentration of PUFAs is increased 4-fold (Fraser *et al.* 2003). However, clinical studies on neuronal effects of PUFAs have produced conflicting results, possibly because of different administration times and doses (Taha *et al.* 2010a). To unveil the neuronal targets of PUFAs is critical both to understand PUFAs' anti-excitability mechanism, and to design future anti-convulsants and analgesics that mimic the PUFA effect and induce reliable clinical anti-excitability effects.

The effect of PUFA on excitability may be mediated by effects on ion channels central for neuronal excitability (Boland & Drzewiecki 2008). For instance, PUFAs inactivate neuronal voltage-gated Na⁺ and Ca²⁺ channels (Vreugdenhil *et al.* 1996; Hong *et al.* 2004), and thereby dampen neuronal activity. Despite the important role of K⁺ channels for excitability, the effect of PUFAs on neuronal voltage-gated K⁺ (K_V) channels is less explored and has shown ambiguous results (Gubitosi-Klug *et al.* 1995; Villarroel & Schwarz 1996; Holmqvist *et al.* 2001; Boland & Drzewiecki 2008).

The M-current is a sustained K^+ current at sub-threshold potentials and is therefore important for setting the negative resting membrane potential of, for instance, hippocampal and dorsal root ganglion (DRG) neurons (Jentsch 2000; Brown & Passmore 2009). At the molecular basis, the M-current conducting channel consists of heteromeric $K_v7.2$ and $K_v7.3$ subunits encoded by *KCNQ2* and *KCNQ3* (Wang *et al.* 1998). These K_v subunits are expressed in many neuronal types, both excitatory and inhibitory neurons (Cooper *et al.* 2001), and in several sub-cellular localizations (Devaux *et al.* 2004). Although the role of M-channels for cellular and network excitability is complex (Vervaeke *et al.* 2006), the most common effect of M-channel activity is reduced neuronal excitability (Brown & Passmore 2009). This is supported by loss-of-function mutations in *KCNQ2* and *KCNQ3* that cause benign familial neonatal seizures (reviewed in Maljevic *et al.* 2008). Moreover, M-channel openers are anti-convulsant and analgesic (Maljevic *et al.* 2008; Grunnet *et al.* 2014).

Arachidonic acid (Schweitzer *et al.* 1990; Behe *et al.* 1992; Villarroel 1994; Yu 1995) and other PUFAs (Behe *et al.* 1992) increase the M-current. PUFA effects on the M-current are however complex. For instance, arachidonic acid-induced increase of M-current was suggested to be mediated by downstream oxygenase metabolites and not by arachidonic acid itself (Schweitzer *et al.* 1990; Yu 1995), while the effect of other PUFAs, not metabolized by oxygenases, seems to be mediated by the PUFAs themselves (Behe *et al.* 1992). PUFA effects also tend to vary with time and from cell to cell in cells with native M-channel expression (Behe *et al.* 1992).

We hypothesized that PUFAs dampen neuronal excitability by directly acting on neuronal M-channels, and hence investigated the effect of PUFAs on mouse DRG neurons endogenously

expressing the M-channel, and on the isolated heteromeric K_v7.2/K_v7.3 channel expressed in *Xenopus laevis* oocytes. We focused on the marine omega-3 (n-3) PUFA docosahexaenoic acid (DHA), because DHA is one of the most abundant PUFAs in the central nervous system (Taha *et al.* 2010a), and found that DHA on the M-channel is the most potent electrostatic K⁺-channel opener described.

Material and Methods

The study conforms with: (Persson 2015).

Patch-clamp on dorsal root ganglion neurons

Seven 7-12 week-old C57BL/6 mice (Scanbur, Denmark) were used. DRG neurons were dissected and cultured for 1-2 days as previously described (Ottoosson *et al.* 2015). Recordings were done at room temperature in whole-cell current-clamp mode. The intracellular solution contained (in mM): 120 K-gluconate, 10 KCl, 1 EGTA, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, pH 7.3. The extracellular solution contained (in mM): 144 NaCl, 2.5 KCl, 10 HEPES, 0.5 MgCl₂, 2 CaCl₂, 10 glucose, pH 7.4. Pipettes were of borosilicate glass and had a resistance of 3-6 MΩ. Small and medium sized DRG neurons were selected. DHA or DHA methyl ester was applied for 90 to 120 s using a pressurized, automated OctaFlow perfusion system (ALA Scientific Instruments, NY, USA). Action potentials were evoked by 50-ms depolarizing pulses from 0.02 to 2 nA in steps of 0.02 to 0.1 nA and the threshold current needed to evoke the first action potential was determined. A liquid-junction potential of −14 mV was corrected for.

Two-electrode voltage clamp on *Xenopus laevis* oocytes

Xenopus laevis oocytes were isolated and maintained as previously described (Bentzen *et al.* 2006). K_v7.2 and K_v7.3 cRNAs were mixed in a 1:1 molar ratio before injection of 50 nl RNA (approximately 1.5 ng of human (h) K_v7.2 (GenBank Acc.No. NM_004518) and 1.5 ng of hK_v7.3 (NM_004519) into oocytes. This channel will hereafter be referred to as the hK_v7.2/3 channel. For the KCNE co-expression experiments, approximately 1.5 ng of hK_v7.2

and 1.5 ng of hK_v7.3 were co-injected with 1 ng cRNA coding for hKCNE1 (NM_000219) or hKCNE2 (NM_172201) into each oocyte. Experiments were conducted 2-5 days after injection at room temperature with two-electrode voltage-clamp, as previously described (Liin *et al.* 2015a). The control solution contained (in mM): 88 NaCl, 1 KCl, 15 HEPES, 0.4 CaCl₂, and 0.8 MgCl₂, pH to 7.4. The holding potential was generally –80 mV with test voltages ranging –110 and +60 mV (2 s durations, 10 mV increments), and tail currents were measured at –30 mV.

Test compounds

4,7,10,13,16,19-*all-cis*-docosahexaenoic acid (DHA), 4,7,10,13,16,19-*all-cis*-docosahexaenoic acid methyl ester (DHA methyl ester), 5,8,11,14,17-*all-cis*-eicosapentaenoic acid (EPA), 9,12,15-*all-cis*-octadecatrienoic acid (ALA), and methyl 9-*cis*-octadecenoic acid (oleic acid) were from Sigma-Aldrich (Stockholm, Sweden). Fatty acids and methyl ester were handled as previously described (Börjesson *et al.* 2010).

Electrophysiological analysis

To quantify the effects on the conductance versus voltage, $G(V)$, curve in *Xenopus* oocytes, tail currents (14 ms after repolarization) were plotted against the test voltage. The following Boltzmann-like expression was fitted to the data

$$G(V) = A / (1 + \exp ((V_{1/2} - V) / s))^4, \quad (1)$$

where $V_{1/2}$ is the midpoint and s the slope factor (shared for control and test compound). $V_{1/2}$ values were compared to quantify the shift in $G(V)$ ($\Delta V_{1/2}$). For illustrative clarity in the figures, the curves are normalized between 0 and 1 and the data points connected by lines.

To quantify the concentration dependence of the DHA-induced $\Delta V_{1/2}$, the following equation was used:

$$\Delta V_{1/2} = \Delta V_{1/2\max} / (1 + (c_{1/2} / c)^{n_H}), \quad (2)$$

where $\Delta V_{1/2\max}$ is the maximal shift, $c_{1/2}$ the concentration causing 50% of the maximal shift, c the concentration of DHA (or H^+), and n_H is the Hill coefficient.

Statistical analysis

Average values are expressed as mean $\pm$ SEM. Mean values for ΔV_m , $\Delta V_{1/2}$ and relative firing threshold were analyzed by a two-tailed one sample t -test where mean values were compared with a hypothetical value of 0 (1 for firing threshold). Following washout, recovery of the DHA effect was analyzed using a two-tailed paired t -test. DHA effects on the hK_v7.2/3 channel, with and without KCNE subunits were compared using one-way ANOVA and Bonferroni's Multiple Comparison Test. $P \leq 0.05$ is considered significant.

Results

DHA hyperpolarizes DRG neurons and affects firing properties

To study the effect of PUFAs on excitability of neurons, we employed the marine omega-3 PUFA DHA (**Fig. 1a**). We added DHA to DRG neurons from mice and measured the effect on the resting membrane potential (V_m) using the patch-clamp technique. The mean resting V_m was -69.1 ± 2.1 mV ($n = 12$). In nine out of 12 DRG neurons, 30 μ M DHA quickly hyperpolarized the resting V_m (**Fig. 1b**). The mean DHA-induced shift in resting V_m in these neurons was -2.4 ± 0.3 mV ($n = 9$, $P = < 0.0001$). This hyperpolarization was reversed when 10 μ M of the M-channel blocker XE991 was applied in the presence of DHA (**Fig. 1b**; $\Delta V_m = +2.7 \pm 0.7$ mV, compared to 30 μ M DHA, $n = 9$, $P = 0.0065$; resting V_m in the presence of DHA + XE991 is not significantly different from the control resting V_m , $P = 0.8$ with paired t-test). The effect of XE991 on resting V_m was similar to previous reports using sensory neurons (Wladyka *et al.* 2008; Du *et al.* 2014). In a subset of DRG neurons (three out of 12), 30 μ M DHA induced a depolarization of the resting V_m by +1, +3 and +3 mV, respectively.

To investigate the DHA effect on the firing properties in DRG neurons, we evoked action potentials in six DRG neurons by using constant depolarizing current pulses of different amplitudes, and applied 30 μ M DHA. Upon DHA application, the resting V_m was hyperpolarized (**Fig. 1c**) in all six neurons (by -0.5 to -4 mV). Furthermore, the threshold current to evoke action potentials was increased (**Fig. 1c**) in all six neurons; in two of the neurons, action potentials could not be evoked in the presence of DHA and in the remaining four, 30 μ M DHA increased the threshold current by a factor of 1.29 ± 0.06 ($n = 4$, $P = 0.02$). The DHA effects in DRG neurons had slow and incomplete recovery upon perfusion with

control solution. In summary, DHA exerts a dampening effect on the excitability of DRG neurons, presumably through an augmentation of the M-current.

DHA and EPA facilitate opening of the hK_V7.2/3 channel expressed in *Xenopus oocytes*

To study the effect of DHA on the isolated M-channel, we co-expressed human K_V7.2 and K_V7.3 subunits in *Xenopus laevis* oocytes to form heteromeric hK_V7.2/3 channels. In line with DHA-induced augmentation of the M-current, extracellular application of 70 μ M DHA shifted the $G(V)$ curve of the hK_V7.2/3 channel towards more negative voltages (**Fig. 2a-b**; $\Delta V_{1/2} = -9.3 \pm 1.7$ mV, $n = 5$, $P = 0.005$). Similar to DRG neurons, the onset of the DHA effect in *Xenopus* oocytes was quick (inset in **Fig. 2b**). DHA concentrations of 7 μ M and higher induced significant $G(V)$ shifts and 50% of the maximum $G(V)$ shift was achieved by 10.2 ± 5.5 μ M DHA (**Fig. 2c**). 70 μ M DHA had no significant effect on the maximum conductance ($P = 0.7$ with paired t-test, $n = 5$). For 70 μ M DHA, the recovery of $\Delta V_{1/2}$ was $52 \pm 8\%$ after washing with a control solution supplemented with 100 mg/L albumin. To test whether the ability to facilitate the hK_V7.2/3-channel opening was unique to DHA or applicable also to other PUFAs, we tested the effect of the PUFAs α -linolenic acid and eicosapentaenoic acid (ALA and EPA respectively, **Fig. 1a**). ALA and EPA induced a similar $G(V)$ shift as DHA: 70 μ M ALA and EPA shifted the $G(V)$ curve of the hK_V7.2/3 channel by -7.4 ± 0.5 mV ($n = 5$, $P = 0.0001$) and -11.3 ± 1.2 mV ($n = 4$, $P = 0.003$), respectively (**Fig. 2d-e**).

PUFA methyl ester and monounsaturated fatty acid do not affect the hK_V7.2/3 channel expressed in *Xenopus oocytes*

Previous studies have given contradicting results on whether uncharged DHA analogues dampen neuronal firing (Xiao & Li 1999; Taha *et al.* 2013). The structure of the uncharged DHA methyl ester is identical to the structure of DHA, except that the DHA methyl ester has a methyl group coupled to the carboxyl group, which renders the molecule uncharged (**Fig. 1a**). In contrast to DHA, 70 μ M DHA methyl ester did not facilitate the opening of the hK_V7.2/3 channel expressed in *Xenopus* oocytes (**Fig. 3a-b**; $\Delta V_{1/2} = -1.0 \pm 0.5$ mV, $n = 4$, $P = 0.1$). Likewise, it did not open M-channels to hyperpolarize DRG neurons; in two out of seven DRG neurons tested, 30 μ M DHA methyl ester slowly induced a depolarization of the resting V_m by +2 and +3 mV, respectively, and in the remaining five cells, 30 μ M DHA methyl ester had no effect on the resting V_m (-0.5 ± 0.3 mV ($n = 5$, $P = 0.2$)).

The lack of effect of the DHA methyl ester on $V_{1/2}$ of the hK_V7.2/3 channel may be because a charged moiety of the fatty acid is required to affect the hK_V7.2/3 channel, as has previously been suggested for the related hK_V7.1 channel and the *Drosophila Shaker* K⁺ channel (Börjesson *et al.* 2010; Liin *et al.* 2015a). If the negative charge of the carboxyl head of DHA is critical to facilitate the hK_V7.2/3 channel opening, we would expect the DHA effect to depend on carboxyl charge protonation, and thus to be pH dependent. To test this hypothesis, we measured the ability of DHA to facilitate hK_V7.2/3 channel opening in extracellular pH ranging from 6.5 to 10; the DHA-induced $G(V)$ shift of the hK_V7.2/3 channel was enhanced at high pH (**Fig. 3c**; $\Delta V_{1/2}$ at pH 9 = -24.7 ± 3.9 mV, $n = 8$, $P = 0.0004$), in which a large fraction of DHA molecules are deprotonated and negatively charged. In contrast, the ability of DHA to shift the $G(V)$ of the hK_V7.2/3 channel was abolished at pH 6.5 (**Fig. 3d**; $\Delta V_{1/2}$ at pH 6.5 = -0.8 ± 0.8 mV, $n = 3$, $P = 0.4$), in which a large fraction of DHA molecules are protonated and uncharged. The pH-response relationship for 70 μ M DHA is summarized in **Figure 3e**.

Previous studies have also provided contradicting indications on whether only PUFAs dampen excitability or if also monounsaturated fatty acids act anti-excitabile (Bendahhou *et al.* 1997; Voskuyl *et al.* 1998; Hong *et al.* 2004; Taha *et al.* 2010b; Bandero *et al.* 2013; Taha *et al.* 2013). Oleic acid (**Fig. 1a**) is an abundant monounsaturated fatty acid in the nervous system (Fraser *et al.* 2003). Oleic acid has identical acyl chain length to ALA (18 carbons) but is lacking two of the double bonds present in ALA. In contrast to the three tested PUFAs, 70 μ M monounsaturated oleic acid did not facilitate the opening of the hK_V7.2/3 channel expressed in *Xenopus* oocytes (**Fig. 3f-g**; $\Delta V_{1/2} = +0.4 \pm 0.5$ mV, $n = 4$, $P = 0.5$).

KCNE co-expression does not alter DHA sensitivity of the hK_V7.2/3 channel expressed in *Xenopus* oocytes

Auxiliary KCNE β -subunits are critical for proper cardiac and epithelial function of the related K_V7.1 channel (Liin *et al.* 2015b). KCNE subunits are widely distributed and also expressed in the nervous system (McCrossan & Abbott 2004). KCNEs have therefore been speculated to be regulatory subunits of the native M-channel. For instance, mRNA expression patterns of KCNE2, K_V7.2 and K_V7.3 are largely similar in the mouse brain (Tinel *et al.* 1998; Tinel *et al.* 2000). It remains however to be determined whether the K_V7.2/3 channel and KCNE subunits form functional neuronal complexes *in vivo*. PUFA effects on the hK_V7.1 channel are altered by KCNE co-expression (Liin *et al.* 2015a; Moreno *et al.* 2015). We therefore tested whether KCNE co-expression altered PUFA effects on the hK_V7.2/3 channel. For these experiments, hK_V7.2/3 was co-expressed with either hKCNE1 or hKCNE2 in *Xenopus* oocytes because these KCNE subunits have previously been shown to modulate the activity of K_V7.2/3 in heterologous expression systems (Yang *et al.* 1998; Tinel *et al.* 2000).

In line with previous reports (Yang *et al.* 1998; Tinel *et al.* 2000), co-expression of hK_V7.2/3 and hKCNE1 generated currents with slowed activation kinetics (**Fig. 4a**) while co-expression of hK_V7.2/3 and hKCNE2 generated fairly hK_V7.2/3-like currents (**Fig. 4c**).

hK_V7.2/3+hKCNE1 currents were contaminated by currents likely generated by endogenous K_V7.1 and injected hKCNE1 at depolarized voltages (Yang *et al.* 1998), at which the K_V7.1/KCNE1 channel is active. Therefore, currents generated by voltage steps more positive than 0 mV were discarded in our analysis of hK_V7.2/3+hKCNE1. As for hK_V7.2/3 alone, 70 μ M DHA increased hK_V7.2/3+hKCNE1 and hK_V7.2/3+hKCNE2 current amplitude at negative voltages and shifted the $G(V)$ curve towards more negative voltages (**Fig. 4a-d**; for hKCNE1: $\Delta V_{1/2} = -8.2 \pm 2.3$ mV, $n = 4$, $P = 0.04$; for hKCNE2: $\Delta V_{1/2} = -4.6 \pm 0.5$ mV, $n = 5$, $P = 0.0006$). Thus, co-expression of hK_V7.2/3 with hKCNE1 or hKCNE2 in *Xenopus* oocytes did not significantly alter the ability of DHA to facilitate opening of the hK_V7.2/3 channel ($P > 0.05$ with ANOVA).

Discussion

In this study, we provide evidence that PUFAs open neuronal M-channels, thereby dampening neuronal excitability. We found that DHA hyperpolarized the resting membrane potential of DRG neurons and increased the firing threshold current in these neurons. The PUFAs DHA, ALA, and EPA facilitated opening of the hK_v7.2/3 channel (=M-channel) expressed in *Xenopus* oocytes by shifting the *G*(*V*) curve towards more negative voltages. In contrast to the facilitating effects found for PUFAs, the uncharged PUFA analogue DHA methyl ester and the monounsaturated oleic acid were ineffective on the M-channel expressed in *Xenopus* oocytes. DHA methyl ester also failed to hyperpolarize the resting membrane potential of DRG neurons. Co-expression of KCNE1 or KCNE2 did not significantly alter the DHA effect on the M-channel.

The concentration of free PUFAs in serum, and likely in cerebrospinal fluid, is around 10-50 μ M, but may be as high as 135 μ M (Conquer & Holub 1998; Fraser *et al.* 2003; Brouwer *et al.* 2006). The majority of the circulating ‘free’ PUFA is expected to be bound to serum albumin (Cistola & Small 1991). Cellular membranes however compete with albumin for fatty acid binding (Cistola & Small 1991). Some membranes even have specific fatty acid-binding proteins that facilitate the uptake of fatty acids (van der Vusse 2009). Under conditions with elevated serum PUFAs, as seen during ketogenic-diet treatment, the fraction of PUFAs associated with cellular membranes increases dramatically (Cistola & Small 1991). The albumin concentration in cerebrospinal fluid is considerable lower than in serum (Seyfert *et al.* 2004). This complex interplay between PUFAs and proteins/membranes makes it hard to estimate how much of the circulating free PUFAs that are available to affect peripheral and central neurons and to determine what physiological PUFA concentration to relate our work

to. For simplicity, we therefore assume that most of the free circulating PUFAs are available to affect neurons.

In this work, we found that 30 μ M DHA hyperpolarized the resting membrane potential of DRG neurons by -2.4 mV and shifted the $G(V)$ curve of the M-channel in *Xenopus* oocytes by -9 mV (deduced from **Fig. 2c**). Previous computer simulations suggest that quantitatively similar small alterations (-1 to -5 mV) in the resting membrane potential of neurons (Tigerholm *et al.* 2012) and in the voltage dependence of ion channels (Thomas *et al.* 2009; Tigerholm *et al.* 2012) are effective means to reduce neuronal excitability and prevent epileptic activity. These computer simulations in combination with our experimental findings in DRG neurons and on isolated M-channels suggest that PUFA-induced augmentation of the M-current contributes to the anti-excitability effect of PUFAs. This is further supported by a comparison of the PUFA effects found in the present work and previously reported effects of the clinically approved M-channel opener retigabine; the PUFA effects on the resting membrane potential and the firing threshold in DRG neurons, and on the voltage dependence of the isolated human M-channel expressed in *Xenopus* oocytes are, on the whole, similar to, but quantitatively smaller than, the effect of retigabine (Main *et al.* 2000; Zheng *et al.* 2013; Du *et al.* 2014). Our finding that DHA methyl ester and oleic acid were ineffective suggests that possible anti-excitability effects of PUFA methyl esters and monounsaturated fatty acids are not mediated via the M-channel. In a subset of DRG neurons, we found that DHA instead depolarized the resting membrane potential, which would tend to increase excitability. Speculatively, this depolarization may be caused by DHA-induced activation of TRPV1 (Matta *et al.* 2007), which is expressed in small to medium sized DRG neurons (Caterina *et al.* 1997).

We have previously reported that PUFAs facilitate opening of the hK_V7.1 channel (when not co-expressed with the KCNE1 β -subunit) and of the *Shaker* K_V channel, by shifting the $G(V)$ of these channels towards more negative voltages (Xu *et al.* 2008; Börjesson *et al.* 2010; Liin *et al.* 2015a). In similarity to the M-channel findings reported here, PUFA methyl ester and monounsaturated fatty acid were ineffective on those channels. The resemblance in PUFA modulation of these three K_V channels implies a general PUFA binding site and mechanism of action for PUFA-induced augmentation. Thus, PUFAs represent a principally new mechanism for M-channel opening compounds: We have previously suggested PUFAs to interact with the outermost gating charges (positively charged arginines) of the voltage sensor S4 (the fourth transmembrane segment of an ion channel subunit) in K_V channels at the interface between the lipid bilayer and the voltage-sensor domain (S1-S4) (Börjesson & Elinder 2011; Ottosson *et al.* 2014; Liin *et al.* 2015a; Yazdi *et al.* 2016). In contrast, other M-channel openers are suggested to bind to the central core of the voltage-sensor domain (Padilla *et al.* 2009; Peretz *et al.* 2010; Li *et al.* 2013), or to the pore domain (S5-S6) (Xiong *et al.* 2007; Lange *et al.* 2009). However, it should be noted that DHA is much more potent on the M-channel than on the previously investigated K_V channels expressed in *Xenopus* oocytes; the DHA concentration needed to shift the $G(V)$ by -3 mV (in pH 7.4) is only $4\text{ }\mu\text{M}$ for the M-channel, while $36\text{ }\mu\text{M}$ is needed for the K_V1-type *Shaker* channel (Xu *et al.* 2008), and $>70\text{ }\mu\text{M}$ for the cardiac hK_V7.1/hKCNE1 channel (Liin *et al.* 2015a). Hence, PUFAs act on M-channels in a clinically relevant concentration range (Xu *et al.* 2008; Börjesson *et al.* 2010; Tigerholm *et al.* 2012).

We have previously shown that the distribution of charged residues in the extracellular end of S4 impacts PUFA sensitivity of K_V channels; additional positive charges offer stronger electrostatic interaction between the PUFA molecule and the channel (Ottosson *et al.* 2014).

This can, however, not explain the different DHA sensitivities of hK_V7.2/3, hK_V7.1/hKCNE1, and *Shaker* K⁺ channels, because the gating charge profile of the outer end of S4 does not differ between these channels. An alternative explanation is that these channels have different affinities for PUFAs. This is supported by the higher $c_{1/2}$ obtained for the *Shaker* K⁺ channel compared to the hK_V7.2/3 channel (80 μ M (Xu *et al.* 2008) compared to 10 μ M (**Fig. 2c**) under identical conditions). The poor DHA sensitivity of the hK_V7.1/hKCNE1 complex under similar conditions is likely caused by KCNE1-induced protonation of DHA (Liin *et al.* 2015a). Future work that compares the detailed PUFA binding sites in these channels is required for a molecular understanding of differential PUFA sensitivity and differential impact of KCNE co-expression on PUFA sensitivity in K_V channels (Liin *et al.* 2015a; Moreno *et al.* 2015).

To conclude, our data suggest that the neuronal M-channel is one of the important molecular targets of PUFAs, and that PUFA-induced opening of the M-channel contributes to the anti-excitability effect of PUFAs in the nervous system. Thus, anti-excitability PUFA effects *in vitro* and *in vivo* (e.g. Voskuyl *et al.* 1998; Xiao & Li 1999) are likely caused by a combination of effects on for instance voltage-gated Na⁺ and Ca²⁺ (Boland & Drzewiecki 2008) and the M-channel (this study). The molecular details of how PUFAs augment the M-current may function as a framework for the design of future M-channel openers to be used in the treatment of epilepsy and pain.

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Conflict of Interest

The authors declare no competing financial interests.

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Figure legends

Figure 1. Effect of DHA on DRG neurons. (a) Structure of fatty acids and analogues tested. (b) Representative effect of 30 μ M DHA and 10 μ M XE991 on resting V_m in a DRG neuron. Dashed line denotes baseline. (c) Representative effect of DHA on the stimulation current needed to evoke action potentials. The stimulation pulse starts at $t = 2$ ms and continues for 50 ms. Control (blue), 30 μ M DHA (red).

Figure 2. Effect of DHA and EPA on the hK_v7.2/3 channel expressed in *Xenopus* oocytes. (a-b) 70 μ M DHA shifted the $G(V)$ curve of the hK_v7.2/3 channel in negative direction along the voltage axis. Control ($\circ$), DHA ($\bullet$). Arrows in (a) denote currents at -40 mV. The dashed curve in (b) is the control curve shifted -9.6 mV. Inset shows time course of DHA wash-in at -30 mV (steady-state currents at the end of the test pulse). Application of DHA starts at the arrow. (c) Concentration dependence of DHA-induced $\Delta V_{1/2}$. Continuous line is best fit to Eq. 2. $\Delta V_{1/2\max} = -11.0$ mV, $c_{1/2} = 10.2$ μ M, $n_H = 1.1$. Mean $\pm$ SEM. $n = 3-5$. (d-e) 70 μ M ALA or EPA shifted the $G(V)$ curve of the hK_v7.2/3 channel in negative direction along the voltage axis. Control ($\circ$), ALA/EPA ($\bullet$). Dashed curves are the control curves shifted by -8.0 and -10.3 mV, respectively.

Figure 3. Importance of the carboxyl charge and the acyl tail for PUFA effects. (a-b) 70 μ M DHA methyl ester (DHA-me) did not induce $G(V)$ shift of the hK_v7.2/3 channel expressed in *Xenopus* oocytes. Control ($\circ$), DHA-me ($\bullet$). Arrows denote currents at -40 mV. (c-e) pH dependence of DHA effect. Insets in (c) and (d) illustrate how different pH affect protonation of DHA. Red spheres illustrate protons. Dashed curve in (c) is control curve shifted by -18 mV. Continuous line in (e) is best fit of Eq. 2 data for 70 μ M DHA in different pH: $\Delta V_{1/2\max} = -24.4$ mV, $c_{1/2} = 3 \times 10^{-8}$ M = pH 7.5, $n_H = 1.6$. Mean $\pm$ SEM. $n = 3-8$. (f-g)

70 μ M oleic acid (OA) did not induce $G(V)$ shift of the hK_v7.2/3 channel expressed in *Xenopus* oocytes. Control ($\circ$), OA ($\bullet$). Arrows denote currents at -40 mV.

Figure 4. Impact of KCNE co-expression on DHA effects. (a-b) 70 μ M DHA shifted the $G(V)$ curve of the hK_v7.2/3+hKCNE1 channel in negative direction along the voltage axis. Control ($\circ$), DHA ($\bullet$). Arrows denote currents at -50 mV. The dashed curve in (b) is the control curve shifted by -10.0 mV. (c-d) 70 μ M DHA shifted the $G(V)$ curve of the hK_v7.2/3+hKCNE2 channel in negative direction along the voltage axis. Control ($\circ$), DHA ($\bullet$). Arrows denote currents at -50 mV. The dashed curve in (d) is the control curve shifted by -6.0 mV.

Figure 1

Figure 2

Figure 3

Figure 4