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Marine n-3 PUFAs modulate I_{Ks} gating, channel expression, and location in membrane microdomains

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Aims Polyunsaturated fatty n-3 acids (PUFAs) have been reported to exhibit antiarrhythmic properties. However, the mechanisms of action remain unclear. We studied the electrophysiological effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on I_{Ks}, and on the expression and location of K_v7.1 and KCNE1.

Methods and results Experiments were performed using patch-clamp, western blot, and sucrose gradient techniques in COS7 cells transfected with K_v7.1/KCNE1 channels. Acute perfusion with both PUFAs increased K_v7.1/KCNE1 current, this effect being greater for DHA than for EPA. Similar results were found in guinea pig cardiomyocytes. Acute perfusion of either PUFA slowed the activation kinetics and EPA shifted the activation curve to the left. Conversely, chronic EPA did not modify K_v7.1/KCNE1 current magnitude and shifted the activation curve to the right. Chronic PUFAs decreased the expression of K_v7.1, but not of KCNE1, and induced spatial redistribution of K_v7.1 over the cell membrane. Cholesterol depletion with methyl-β-cyclodextrin increased K_v7.1/KCNE1 current magnitude. Under these conditions, acute EPA produced similar effects than those induced in non-cholesterol-depleted cells. A ventricular action potential computational model suggested antiarrhythmic efficacy of acute PUFA application under I_{Kr} block.

Conclusions We provide evidence that acute application of PUFAs increases K_v7.1/KCNE1 through a probably direct effect, and shows antiarrhythmic efficacy under I_{Kr} block. Conversely, chronic EPA application modifies the channel activity through a change in the K_v7.1/KCNE1 voltage-dependence, correlated with a redistribution of K_v7.1 over the cell membrane. This loss of function may be pro-arrhythmic. This shed light on the controversial effects of PUFAs regarding arrhythmias.

Keywords K_v7.1 • KCNE1 • I_{Ks} • PUFAs • DHA • EPA • Lipid rafts

1. Introduction

A large amount of evidence from cellular and animal studies,[1] and from clinical trials,[2–5] suggested that an increased intake of fish oil fatty acids has favourable effects on cardiovascular health. Analyses of these trials concluded that these beneficial effects mainly occur through the prevention of sudden cardiac death, which is often preceded by ventricular arrhythmias, indicating that polyunsaturated fatty n-3 acids (PUFAs) are antiarrhythmic.[3,6] However, not all studies have demonstrated the cardioprotective effects on cardiovascular diseases of PUFA

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consumption. Pro-arrhythmic actions have been described for PUFAs in animal models during acute regional myocardial ischaemia.[7] Moreover, the recent Alpha OMEGA and OMEGA randomized trials, involving patients who suffered myocardial infarction, did not show any improvement in the clinical results following PUFA supplementation,[8,9] and even a deleterious effect due to an increased risk of cardiac death was reported in men with stable angina who were advised to eat fish,[10] or in patients with implantable cardioverter defibrillators.[11] These differences could be explained by the fact that a diet rich in fish oil could be pro- or antiarrhythmic depending on the underlying arrhythmogenic mechanism.[12] In any case, the mechanism underlying the pro- or antiarrhythmic effect after supplementation is thought to be related to the modulation of the cardiac ion channels involved in the genesis and/or maintenance of cardiac action potentials (APs). Indeed, PUFAs decrease I_{Na}, I_{Kur}, I_{to}, I_{Kr}, I_{CaL}, and I_{NCX}, and enhanced I_{Ks} and I_{K1}.[13–19]

K_v7.1 and KCNE1 are the two major pore-forming and ancillary subunits, respectively, responsible for the biophysical properties of I_{Ks} channels.[20] Doolan *et al.* demonstrated that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) do not modify the electrophysiological characteristics of K_v7.1 channels, although DHA, but not EPA, increases the magnitude of the K_v7.1/KCNE1 current in *Xenopus* oocytes, effect mediated by the KCNE1 subunit.[16] In cardiac myocytes from pigs fed with a PUFA enriched-diet, I_{Ks} magnitude was greater than that recorded in myocytes from control animals.[21] However, the precise mechanism by which PUFAs produce these effects on K_v7.1/KCNE1 channels remains to be elucidated.

2. Methods

2.1 Cell transfection

In acute experiments, transfection of pCDNA3.1 KCNQ1/KCNE1 concatemer (human KCNE1 linked to the N-terminus of the human KCNQ1) in COS7 cells was performed with Fugene6, following manufacturer's instructions. In the rest of experiments, cells were transfected with KCNQ1-YFP and KCNE1-CFP.

2.2 Preparation of cell extracts

COS7 cultures were washed twice with ice-cold PBS. Cells were homogenized and cell extracts were prepared as described in Supplementary material online, Methods.

2.3 Western blot analysis

Samples of cell extracts containing equal amounts of protein were boiled in Laemmli SDS loading buffer (250 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 2% β-mercaptoethanol) and size-separated in 7–10% SDS–PAGE. The gels were blotted onto PVDF or nitrocellulose membranes and processed as recommended by the supplier of the antibodies (see Supplementary material online, Methods).

2.4 Lipid rafts

Low-density, Triton-insoluble complexes were isolated as previously described from transfected COS7 cells.[22] Cells were homogenized in 1 mL of 1% Triton X-100 and sucrose was added to a final concentration of 40%. A 5–30% linear sucrose gradient was layered on top and further centrifuged (260 000 *g*) for 20–22 h at 4°C in a Beckman SW41 rotor. Gradient fractions (1 mL) were collected from the top and analysed by western blotting.

2.5 Electrophysiological recordings

Currents were recorded using the perforated amphotericin B or whole-cell patch-clamp technique with an Axopatch 200B amplifier (Axon instruments) as described.[23,24] The investigation conforms to the NIH guidelines (Guide for the Care and Use of Laboratory Animals; NIH publications number 23–80) revised in 2011; and from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the University of Milano-Bicocca ethics review board. Adult Dunkin-Hartley guinea pig was anesthetized by 100 mg/kg sodium thiopental (Sigma Aldrich, St Louis, MO, USA) and euthanized by cervical dislocation. Guinea pig ventricular myocytes were isolated as previously described.[25] Membrane cholesterol depletion was achieved by methyl-β-cyclodextrin (MβCD) treatment (2 μM).

2.6 Statistical analysis

Data are presented as mean values ± SEM. Paired or unpaired Student's *t*-test or repeated-measures ANOVA followed by the Bonferroni test. A value of $P < 0.05$ was considered statistically significant.

2.7 Computational modelling

The K_v7.1/KCNE1 current was described by a Markov model, whose transition rates were set by fitting the experimental recordings obtained in the different conditions. The current formulation was then inserted into the O'Hara–Rudy model of human ventricular action potential. An expanded description is available in Supplementary material online, Methods.

3. Results

3.1 EPA and DHA increase K_v7.1/KCNE1 current

EPA or DHA concentration (20 μM) used in this study was selected on the basis of the free fatty acid levels in the plasma of six patients included in the SOFA trial[26] who were taking 2 g/day fish oil. They exhibited concentrations of free EPA and DHA of ~10 μM (5.0–16.4 μM) that accumulate in atria at expenses of arachidonic acid from phospholipids. *Figure 1A* shows K_v7.1/KCNE1 recordings obtained after applying 12 s pulses from a holding potential of −80 to +60 mV in the absence and in the presence of EPA or DHA. Both PUFAs increased the current by 37.3 ± 6.2% ($P < 0.05$, $n = 13$) and 82.8 ± 27.0% ($P < 0.05$, $n = 13$) for EPA and DHA, respectively. The increase induced by EPA was time-dependent, this effect being greater after longer depolarizing times (3.6 ± 6.2, 28.4 ± 5.3, and 37.3 ± 6.2% after 1.5, 5.5 and 12 s, respectively, $P < 0.05$, $n = 5–13$). However, the DHA-induced increase was not time-dependent (91.8 ± 19.3, 92.7 ± 23.8, and 82.8 ± 26.9%, when measured after 1.5, 5.5, or 12 s pulses, respectively, $P > 0.05$, $n = 4–13$).

The K_v7.1/KCNE1 activation kinetics was described by a biexponential process. *Figure 1B* shows the normalized currents obtained under control conditions and after exposure to EPA or DHA. Under control conditions, the activation time constants arose mean values of $\tau_f = 737 \pm 53$ ms and $\tau_s = 4604 \pm 542$ ms ($n = 16$). EPA slowed the activation kinetics due to an increased contribution of the slow component to the activation process [$A_s/(A_s + A_f) = 0.23 \pm 0.04$ vs. $A_s/(A_s + A_f) = 0.64 \pm 0.05$, $P < 0.05$, $n = 8$; *Figure 1B* inset]. The time constants were not modified after perfusing cells with EPA ($\tau_f = 653 \pm 56$ ms and $\tau_s = 4106 \pm 796$ ms, $P > 0.05$, $n = 8$). Similar results in the activation kinetics were observed for DHA [$A_s/(A_s + A_f) = 0.19 \pm 0.04$ vs.

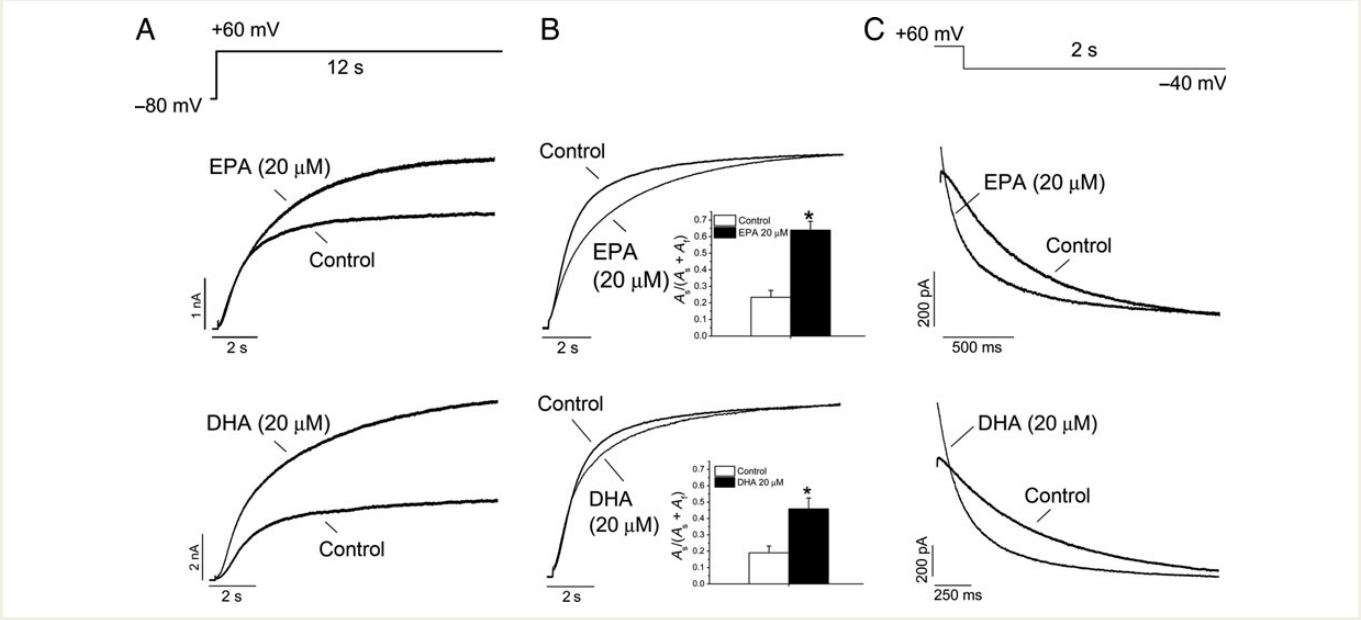

Figure 1 EPA and DHA effects on K_v7.1/KCNE1 currents. (*A*) Original traces of K_v7.1/KCNE1 current obtained in the absence and in the presence of EPA or DHA. (*B*) Normalized traces. The inset represents the plot of the $A_f/(A_f + A_s)$ in control and in the presence of either PUFA. (*C*) Tail currents recorded at −40 mV after 5.5 s pulses to +60 mV in the absence and in the presence of each PUFA.

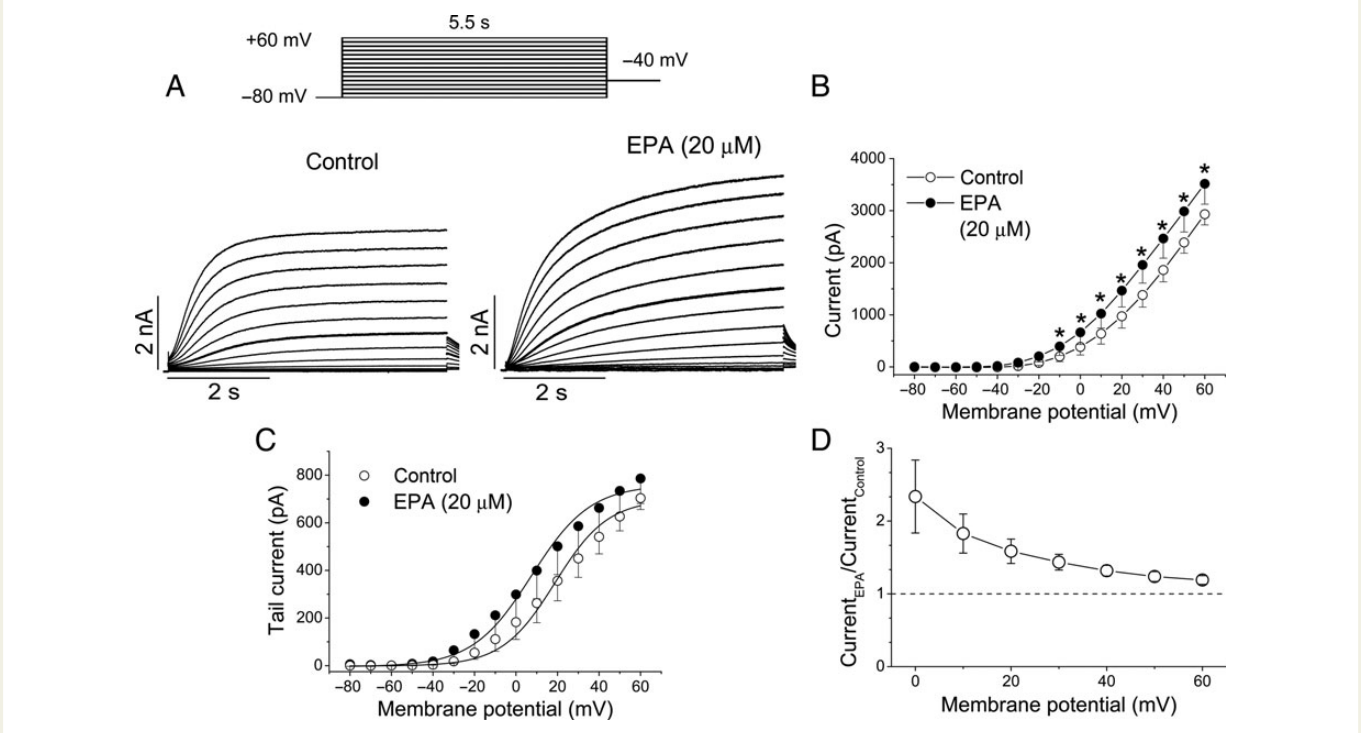

Figure 2 EPA acute effects on K_v7.1/KCNE1 channels. (*A*) Traces obtained after applying the pulse protocol shown in the top in the absence and in the presence of EPA. (*B*) *I*–*V* relationship obtained under control conditions and after perfusion with EPA. (*C*) Activation curves obtained under control conditions and after perfusion with EPA. (*D*) Graph showing the ratio between the current obtained in the presence of EPA and that under control conditions.

$A_s/(A_s + A_f) = 0.46 \pm 0.07, P < 0.05, n = 8$]. However, DHA accelerated the fast component of activation ($\tau_f = 737 \pm 53$ ms vs. $\tau_f = 592 \pm 52$ ms, $P < 0.05, n = 8$).

Tail currents were elicited upon repolarization to −40 mV after each voltage step. An increase in the tail current magnitude of 26.5 ± 14.6% ($P < 0.05, n = 14$) and 27.3 ± 13.3% ($P < 0.05, n = 9$) after exposure

to EPA or DHA was observed (*Figure 1C*). Under control conditions, the deactivation exhibited monoexponential kinetics. However, in the presence of either PUFA, this kinetics became biexponential (see Supplementary material online, *Table S1*).

Figure 2A shows current recordings obtained in the absence and presence of EPA when applying 5.5 s pulses from −80 to +60 mV in 10 mV steps from a holding potential of −80 mV. EPA (20 μM) increased the amplitude of the current at all membrane potentials tested positive to −10 mV (*Figure 2B*) and shifted the V_{mid} of the activation curve towards more negative potentials (+22.3 ± 6.6 vs. +13.5 ± 5.5 mV, $P < 0.05$, $n = 5$) without modifying the slope (16.7 ± 1.3 vs. 18.6 ± 1.7 mV, $P > 0.05$, $n = 5$; *Figure 2C*). *Figure 2D* shows the relative current in the presence of EPA vs. the membrane potential. The maximal increase occurred at ∼0 mV. This effect might be due to the negative shift of the activation curve, suggesting that the primary mechanism of the increased magnitude of K_v7.1/KCNE1 may be due to an effect on channel gating. Similar qualitative effects were produced by DHA (see Supplementary material online, *Figure S1*).

It has been described that the slow gating of I_{Ks} is likely due to the fact that KCNE1 accessory subunit slows the movement of the voltage sensors. To determine that the concatemeric construction does not interfere with the normal properties of I_{Ks} and also to analyse the effects of EPA and DHA, a series of experiments in which the currents were recorded in COS7 cells co-transfected with KCNQ1-YFP and KCNE1-CFP were performed. As shown in Supplementary material online, *Figure S2*, the effects produced by EPA/DHA on I_{Ks} were similar than those observed in cells transfected with the concatemer KCNQ1/KCNE1 subunits. The only difference observed was that the activation kinetics of the K_v7.1/KCNE1 current became monoexponential (see Supplementary material online, *Tables S2–S4*).

Effects of PUFAs were also evaluated on native I_{Ks} recorded in guinea pig ventricular myocytes at 36°C. I_{Ks} was recorded at +20 mV under I_{CaL} and I_{Kr} blockade by nifedipine (10 μM) and E-4031 (5 μM), respectively. A total of 14 myocytes were analysed. In 11 myocytes, DHA (10 μM) increased I_{Ks} amplitude (+37.7 ± 6%, $P < 0.05$) and accelerated its deactivation at −40 mV ($t_{1/2}$ −19.1 ± 8%, $P < 0.05$, see Supplementary material online, *Figure S3*). However, in 5 of 11 myocytes, during long-time recordings, the initial increase of I_{Ks} was followed by a progressive decrease in the current. Moreover, in three myocytes, exposure to 10 μM DHA was directly followed by a decline in I_{Ks} amplitude (data not shown).

3.2 Acute vs. chronic effects of EPA and DHA on K_v7.1/KCNE1 current

An increased consumption of PUFAs leads to increased blood and tissue levels of PUFAs.[26] However, the relative contribution of short- and long-term administration on the electrophysiological effects on ion currents has not been determined. Since we wished to analyse and relate the electrophysiological effects with the effects on the levels of expression and also targeting to the lipid rafts, the same conditions were used in all these experiments: COS7 were co-transfected with KCNQ1-YFP and KCNE1-CFP for 48 h with 20 μM EPA/DHA in serum-free medium. Controls of these experiments were COS7 cells cotransfected with KCNQ1-YFP and KCNE1-CFP incubated for 48 h in serum-free medium without EPA/DHA. To differentiate the acute from the possible chronic effects of EPA and DHA, three different approaches were used. First, we analysed the electrophysiological properties of K_v7.1/KCNE1 currents. Incubation with EPA did not alter the activation kinetics of K_v7.1/KCNE1 (*Figure 3*; $\tau_f = 649 \pm 42$ ms and $\tau_s = 5151 \pm 788$ ms vs. $\tau_f = 771 \pm 89$ ms and $\tau_s = 5112 \pm 1091$ ms, $P > 0.05$, $n = 9–18$, for control conditions and chronic EPA, respectively). DHA accelerated the activation τ_s ($\tau_f = 649 \pm 42$ ms and $\tau_s = 5151 \pm 788$ ms vs. $\tau_f = 587 \pm 74$ ms, $P > 0.05$ and $\tau_s = 3100 \pm 4291$ ms, $P < 0.05$, $n = 9–18$, for control conditions and chronic DHA, respectively, see Supplementary material online, *Figure S4*). The deactivation kinetics was faster than under control conditions, becoming biexponential ($\tau_f = 266 \pm 40$ ms and $\tau_s = 847 \pm 108$ ms for chronic EPA, $n = 9$; and $\tau_f = 296 \pm 26$ ms and $\tau_s = 1235 \pm 171$ ms in serum-free control conditions, $n = 18$), but slower than for acute EPA ($P < 0.05$), without changes in the $A_f/(A_f + A_s)$ relation. Similar effects on the deactivation were observed with chronic DHA ($\tau_f = 266 \pm 40$ ms and $\tau_s = 802 \pm 83$ ms for chronic DHA, $n = 12$; and $\tau_f = 296 \pm 26$ ms and $\tau_s = 1235 \pm 171$ ms in serum-free control conditions, $n = 18$). Chronic EPA or DHA did not modify the current magnitude (*Figure 3C* and see Supplementary material online, *Figure S4C*). EPA induced a positive shift of the activation curve ($V_{mid} = 21 \pm 3$ vs. 31 ± 3 mV, $P < 0.05$, $n = 14–7$, for control and chronic EPA, respectively; *Figure 3D*). However, DHA shifted the activation curve to negative potentials ($V_{mid} = 21 \pm 3$ vs. 13 ± 4 mV, $P < 0.05$, $n = 14–12$, for control and chronic DHA, respectively). Thus, chronic EPA and DHA produce different effects in the channel voltage-dependence.

Chronic treatment with EPA and DHA, but not with α-linolenic acid, decreases the protein levels of K_v1.5 in L-cells.[19] Thus, our second approach was to analyse whether chronically applied EPA and DHA modified the expression pattern of K_v7.1 and KCNE1 subunits. To that end, COS7 cells were transfected with K_v7.1-YFP alone or together with KCNE1-CFP, and the levels of K_v7.1 and KCNE1 were measured by western blot. Under both experimental conditions, EPA reduced the protein levels of K_v7.1 in a concentration-dependent manner (*Figure 4A*), but not those of KCNE1 (*Figure 4C*). Internalization and stability of K_v7.1 is regulated by ubiquitylation,[27] and PUFAs are able to modify the activity of the proteasome. Thus, the decrease in K_v7.1 protein level might be due to a higher degree of K_v7.1 degradation via proteasome. Hence, a series of experiments in the presence of 2 μM MG132 (proteasome inhibitor) were performed. Under these experimental conditions, EPA did not produce any change in the protein levels of K_v7.1, thus suggesting that EPA induces degradation of K_v7.1 via proteasome (*Figure 4B*). Similar results were obtained for DHA (see Supplementary material online, *Figure S5*).

K_v7.1 and KCNE1 subunits partially target to lipid rafts in HEK-293 cells and ventricular myocytes.[22,28] Dietary PUFAs incorporate into the cell membrane and, by altering the lipid composition and protein distribution of lipid raft microdomains, may affect ion channel function.[21] Therefore, the third approach was to analyse whether EPA and DHA modified the location of K_v7.1 and/or KCNE1 in lipid rafts. *Figures 4D* and see Supplementary material online, *Figure S5D* show the distribution of K_v7.1 and KCNE1 over the sucrose gradient under control conditions and after incubation with EPA or DHA for 48 h. Both PUFAs altered the buoyancy of cholesterol-enriched domains as indicated by a redistribution of caveolin thorough the gradient. Concomitantly, the localization of K_v7.1 and KCNE1 was also varied, indicating different channel localization in membrane microdomains. A spatial redistribution of K_v7.1 and KCNE1 in the membrane surface can account for some of the electrophysiological changes observed on K_v7.1/KCNE1.

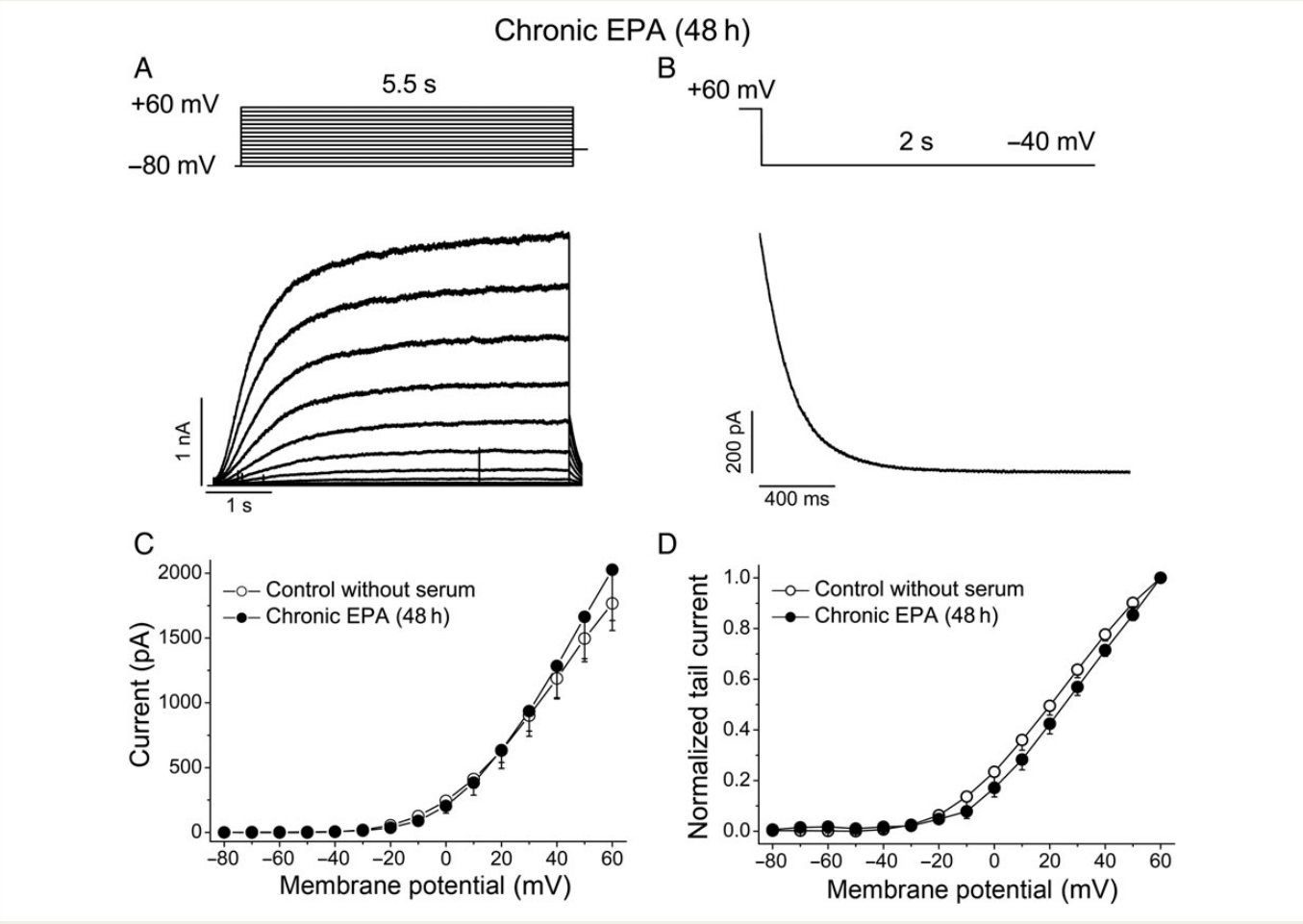

Figure 3 EPA chronic effects on K_v7.1/KCNE1 channels. (*A*) Currents are shown for pulses from −80 mV to voltages between −80 and +60 mV in steps of 10 mV. (*B*) Original tail currents recorded at −40 mV after a 5.5 s depolarization to +60 mV. (*C*) *I*–*V* relationship obtained after measuring the current after 5.5 s. (*D*) Activation curves of K_v7.1/KCNE1 current obtained after representing the maximum tail current amplitude vs. the previous step potential recorded in cells incubated for 48 h with EPA.

3.3 Effects of cholesterol depletion on K_v7.1/KCNE1 current

Association with lipid rafts is an important signalling mechanism for ion channel function and changes and disruption of these microdomains modify the activity of ion channels.[29]

Because PUFAs modified the localization of K_v7.1 channels in lipid microdomains, we analysed the electrophysiological consequences of the disruption of these structures (by removing cholesterol from the membrane with MβCD) on K_v7.1/KCNE1. COS7 cells were sequentially perfused with different external solutions in the following order: (i) normal external solution; (ii) 10 mM MβCD; (iii) 10 mM MβCD + 20 μM EPA, and (iv) drug-free external solution with 10 mM MβCD. MβCD triggered an increase in the K_v7.1/KCNE1 current at potentials positive to +30 mV and a decrease in the current at potentials negative to +30 mV (*Figure 5A–D*). Concomitantly, a positive shift of the activation curve approximately +23 mV ($P < 0.05$, $n = 4$) was observed (*Figure 5D* and *E*; see Supplementary material online, *Table S5*). Under these conditions, perfusion with EPA produced similar effects than those observed in cells non-treated with MβCD. Thus, (i) an increase in the current at potentials negative to +40 mV; (ii) no changes in the midpoint of the activation curve compared with that obtained in the presence of MβCD; and (iii) an increased slope factor. EPA effects were accompanied by the typical slowing of the activation kinetics that was due to the abolishment of the fast component of the activation process (see Supplementary material online, *Table S6*) and the appearance of a second component in the deactivation processes, becoming again biexponential. EPA effects produced in cholesterol-depleted cells with MβCD were reversible, not only in the magnitude of the current, but also in the kinetics and in the activation curve (*Figure 5*).

3.4 Computational modelling of the experimental results

A Markov model of the human K_v7.1/KCNE1 channel[30,31] was optimized to reproduce PUFA acute effects observed in K_v7.1/KCNE1, within the same model structure, by adjusting the parameters governing the transitions between the different channel states (see Supplementary material online, *Figures S6–S8*) and, for the case of chronic exposure to EPA, the maximal current conductance (see Supplementary material online, *Figure S6D*). Model simulations of K_v7.1/KCNE1 current reproduced accurately the experimental current traces under voltage clamp (see Supplementary material online, *Figure S6*) as well as current–voltage and tail current–voltage relationships (see Supplementary material

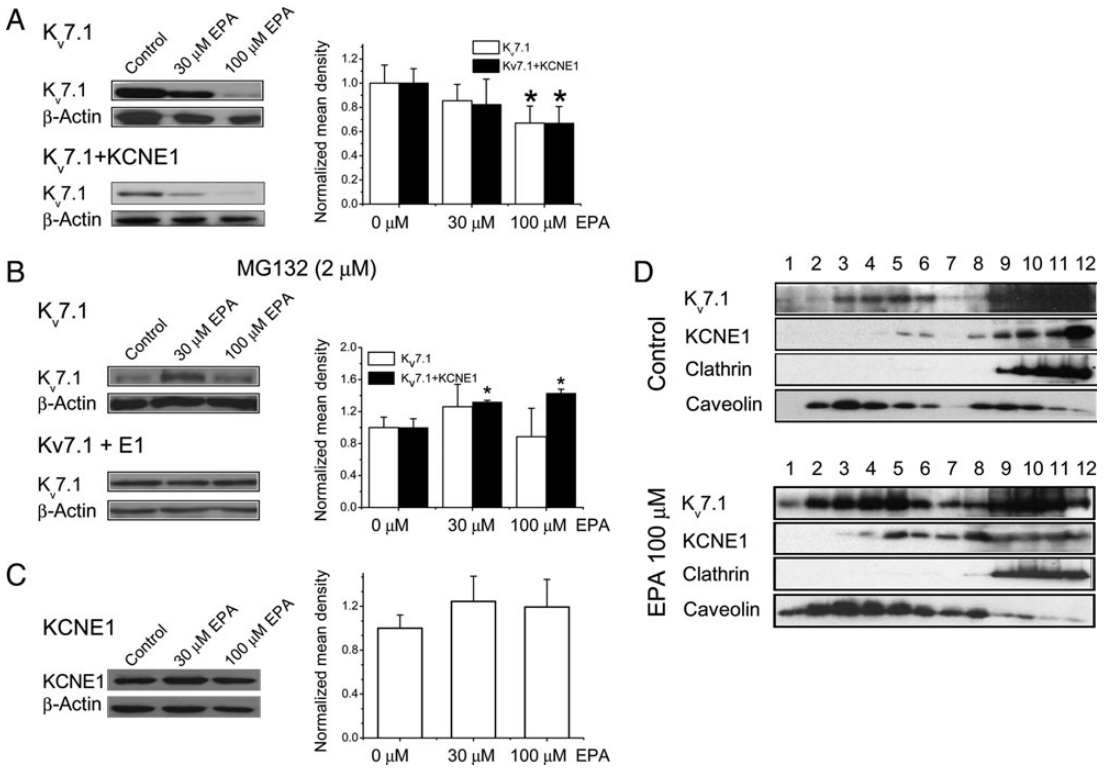

Figure 4 EPA decreases the K_v7.1 protein expression in COS7 cells. (*A*) Left: representative western blots illustrating treatments with EPA. Note that EPA induces dose-dependent reductions of K_v7.1 protein. Cellular lysates were prepared from COS7 cells transiently expressing K_v7.1 and incubated for 48 h with 30 and 100 μM EPA. Samples were subjected to SDS–PAGE, transferred to PVDF, and probed with an anti-K_v7.1 antibody. Right: Bar graph summarizing densitometry measurements used to compare protein levels for the treatments of EPA for 48 h. β-Actin levels were used as a loading control ($n = 3$ or 4; $^*P < 0.05$). (*B*) Representative western blots and graph showing the effects of EPA (0, 30, and 100 μM) on K_v7.1 in cells transfected with K_v7.1 or K_v7.1/KCNE1 in the presence of MG132 (proteasome inhibitor). (*C*) Representative western blots and graph showing the effects of EPA (0, 30, and 100 μM) on K_v7.1 in cells transfected with KCNE1. (*D*) Sucrose density gradient fractions of cells expressing K_v7.1 and KCNE1 in the absence or the presence of EPA. While caveolin indicated floating lipid rafts with low-density, clathrin-labelled non-raft fractions. K_v7.1 and KCNE1 co-localized with caveolin in low-buoyant density fractions (fractions 2–6) in control experiments, whereas EPA triggered a wider distribution of proteins (fractions 1–12) and rafts. Pictures are representative images of at least three independent lipid raft extractions analysed by western blot.

online, *Figure S8*). The K_v7.1/KCNE1 current model was incorporated into a biophysically detailed model for human ventricular epicardial cell AP by O'Hara *et al.*,[32] which has a basal AP duration measured at the 90% repolarization (APD_{90}) of 226 ms.

The main effects of the altered parameters during an AP are shown in *Figure 6*, and the effects on the closed zones occupancy are detailed in Supplementary material online, *Figure S9*. PUFAs increased the open state probability of the K_v7.1/KCNE1 channel, leading to an elevated K_v7.1/KCNE1 current during the AP, and slightly shortened the APD_{90} (*Figure 6*). The APD_{90} in the presence of EPA and DHA was 214 and 217 ms, respectively. These observations are also reflected in the simulated S1–S2 restitution (*Figure 6A*). On the contrary, chronic EPA did not significantly altered K_v7.1/KCNE1 when compared with control, and therefore expectedly did not alter the APD_{90}.

PUFA effects on K_v7.1/KCNE1 current were simulated in a condition in which the contribution of K_v7.1/KCNE1 channels to repolarization can be more critical. Indeed, along with their effects on K_v7.1/KCNE1 channels, acute and chronic exposure to PUFAs affects K_v11.1 current. The effects of a graded reduction in K_v11.1 were simulated. K_v7.1/KCNE1 and K_v11.1 current profiles during AP and APD_{90} are illustrated in *Figure 7*. EPA and DHA partially compensated the AP prolongation due to K_v11.1 reduction, whereas the chronic exposure had negligible effects for K_v11.1 reductions up to 60% but led further AP prolongation for 80% K_v11.1 block. The relative shortening of APD_{90} due to EPA in comparison with control APD_{90} was greater as the K_v11.1 block was increased. At a 20% block of K_v11.1, EPA reduced APD_{90} to 93.1%, whereas DHA reduced APD_{90} to 95.3%. At 80% block of K_v11.1, EPA reduced APD_{90} to 83.7%, whereas DHA reduced APD_{90} to 94.1%.

4. Discussion

In this study, we investigated the effects of two n-3 PUFAs from marine origin (EPA and DHA) on K_v7.1/KCNE1 channels expressed in COS7 cells and on I_{Ks} in guinea pig cardiomyocytes. We demonstrated that: (i) at physiological concentrations, both PUFAs increase the magnitude of K_v7.1/KCNE1 current after acute, but not after chronic, exposition, DHA being more potent than EPA. (ii) The response pattern of DHA in ventricular myocytes was complex but mainly consistent with that observed in COS7 cells. (iii) Acute and chronic effects of EPA were time- and voltage-dependent. (iv) Chronic application of either PUFAs reduced the expression of K_v7.1, but not that of KCNE1 due to an

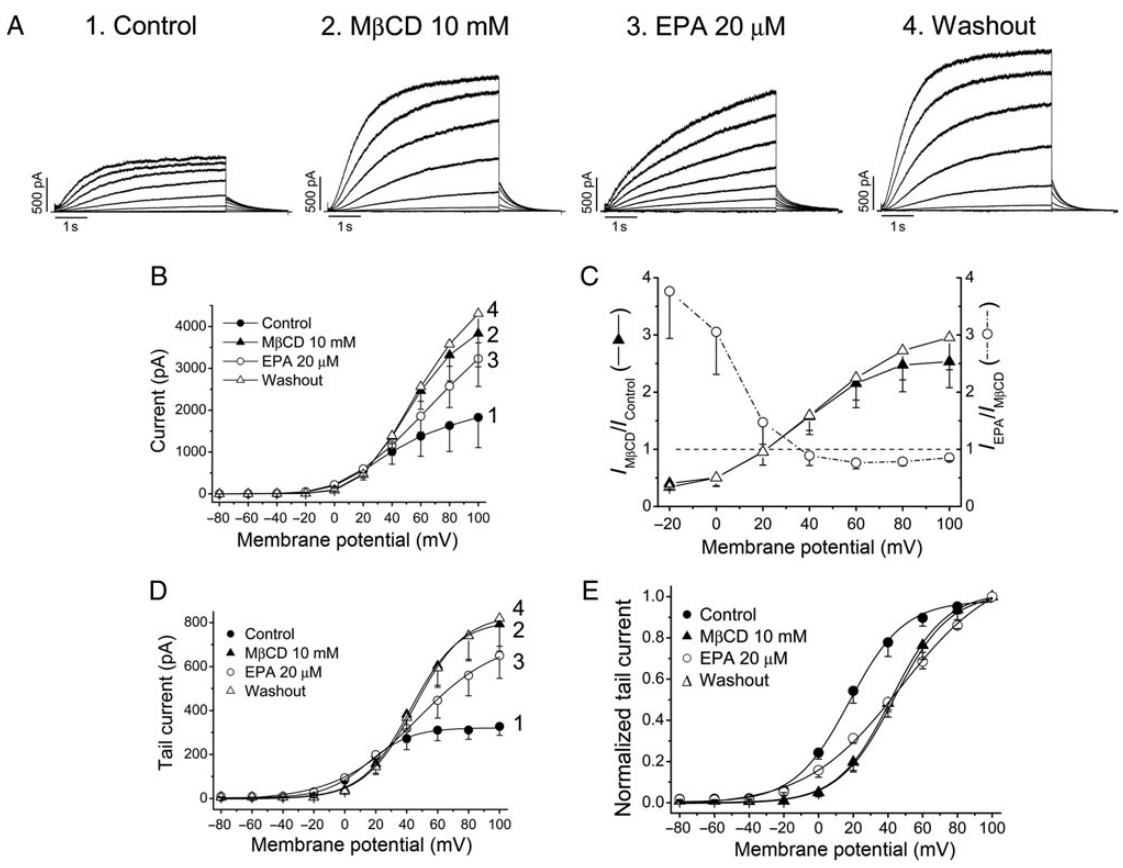

Figure 5 EPA effects in membrane cholesterol-depleted cells. (*A*) Traces of $K_v7.1$/KCNE1 current under control conditions, after perfusion with MβCD for 40 min, with EPA and washout with EPA-free external solution. All recordings were obtained from the same cell. (*B*) *I–V* relationships. (*C*) Graph showing the ratio between the current obtained after perfusion with MβCD (filled triangle) or after washout (open triangle) and that recorded under control conditions, as well as the current recorded in the presence of EPA and after perfusion with MβCD (open circle). (*D*) Activation curves. (*E*) Normalized activation curves.

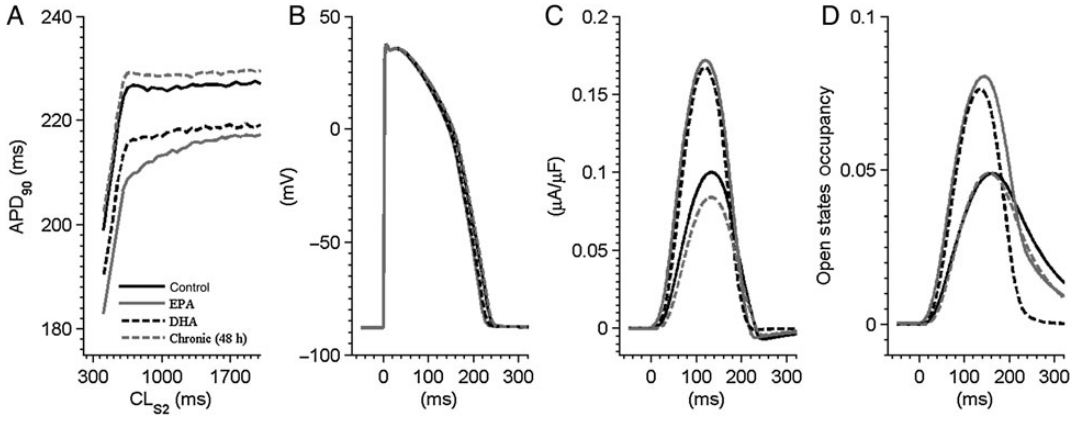

Figure 6 n-3 PUFA effects in the human ventricle O'Hara cell model.[32] (*A*) APD as a function of premature stimulus. (*B*) Action potential. (*C*) I_{Ks} profile during AP, and (*D*) occupancy of open states of the I_{Ks} model (see Supplementary material online, *Figure S9*).

enhanced degradation of $K_v7.1$ via proteasome. (v) Chronic application of either PUFAs modified the $K_v7.1$ channel distribution in membrane microdomains, and (vi) Acute EPA effects on $K_v7.1$/KCNE1 current recorded in cholesterol-depleted cells were similar to those observed without cholesterol depletion, consistent with a direct interaction with the channel during acute application.

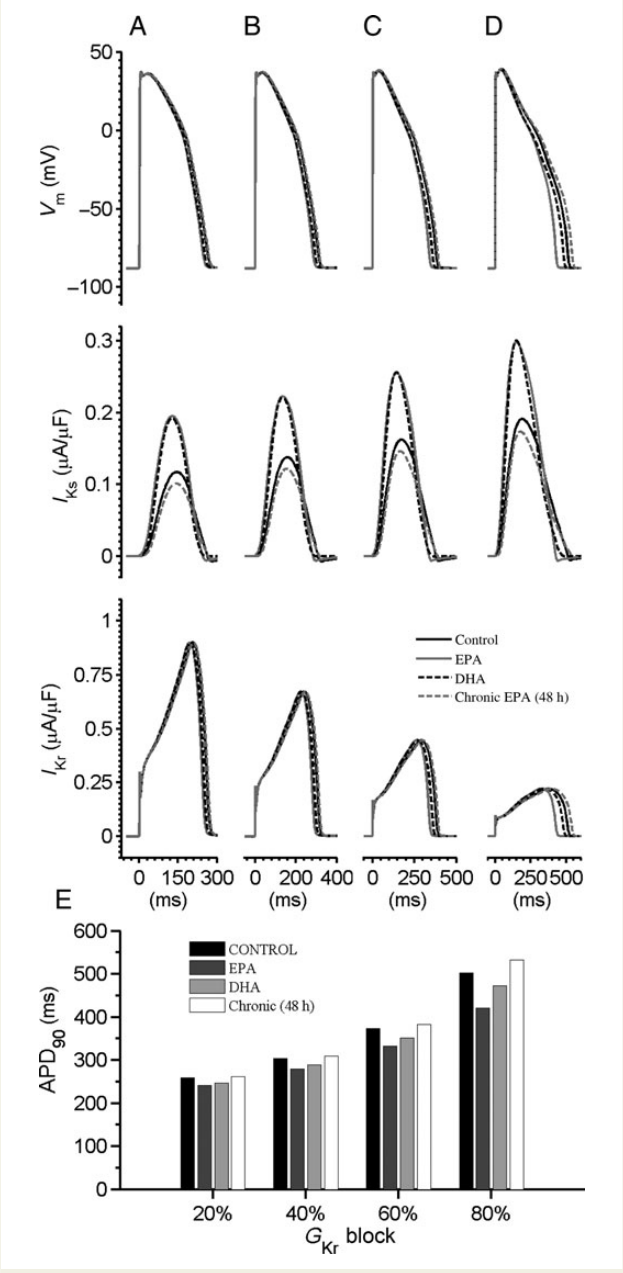

Figure 7 Effect of I_{Kr} block on simulated action potential (first row), I_{Ks} (second row), and I_{Kr} (third row). (*A*) 20% G_{Kr} block. (*B*) 40% G_{Kr} block. (*C*) 60% G_{Kr} block. (*D*) 80% G_{Kr} block. (*E*) Action potential duration (APD_{90}) as a function of G_{Kr} block. G_{Kr}, Maximum conductance of Kr channels.

4.1 Acute and chronic effects of n-3 PUFAs

In this study, we demonstrated that acute perfusion of EPA or DHA increased to a different extent the magnitude of K_v7.1/KCNE1 current. EPA and DHA increased the current and slowed its activation kinetics. Moreover, their effects on the electrophysiological properties of this current exhibited some differences, which are consistent with a direct effect of both PUFAs with these channels. These results can be due to a modification of the interaction between KCNE1 and K_v7.1 subunits, without changes in stoichiometry, since similar effects were observed in cells transfected with the concatemer KCNQ1/KCNE1 construction and cells co-transfected with the two subunits separately. Also, as previously described,[16] neither EPA nor DHA modified the properties of K_v7.1 channels when expressed alone (see Supplementary material online, *Figure S10*). Thus, the presence of KCNE1 subunits is essential for the observed electrophysiological effects of acutely applied PUFAs.

Dietary administration of fish oil leads to the incorporation of PUFAs into the cell membranes.[21] It has been reported that long-term PUFA intake modulates the activity of several cardiac ion channels. However, the effects of long-term administration of PUFAs differ from those observed after acute administration. In this line of evidence, the effects of long-term administration of EPA and DHA (48 h) on COS7 cells expressing K_v7.1/KCNE1 channels were different compared with those produced acutely. Chronic application of EPA or DHA did not increase the current magnitude, but it accelerated the activation process compared with acutely EPA effects. Surprisingly, EPA and DHA shifted the activation curve towards opposite directions, suggesting that the mechanism by which acute or chronic PUFA modulates the electrophysiological properties of K_v7.1/KCNE1 channels is different.

4.2 Direct and indirect effects on K_v7.1/KCNE1 channels

Several hypothesis have been proposed to explain the electrophysiological differences between acute and long-term administration of PUFAs. Some authors explain PUFA effects by a direct interaction between fatty acid and the ion channel. This hypothesis is supported by the observation that substitutions of a single amino acid in the Na_v1.5 channel diminished the inhibitory effect of acutely administered EPA.[33] Our results showing differences between EPA and DHA effects on K_v7.1/KCNE1 channels are consistent with a direct effect between PUFAs and ion channels. However, their effects were not washed-out and thus, we cannot rule out an indirect effect.

Others suggest that PUFA effects are the result of an alteration of the membrane properties rather than a direct interaction with the ion channel. It was reported that PUFAs alter the composition and order of the plasmalema.[12] In this line of evidence, it has been proposed that the potency of PUFAs to inhibit cardiac I_{Na} is correlated with their ability to increase membrane fluidity measured by steady-state fluorescence anisotropy.[34] In addition, changes in cellular redox status, metabolism of phospholipids, and modulation of gene expression are other processes, implying PUFAs that might modulate indirectly ion channel function.[35]

To further explore the mechanisms by which acute and chronic PUFAs exert their actions on K_v7.1/KCNE1 channels, three different approaches were used. First, the effects of chronic administration of EPA were analysed on the expression levels of K_v7.1 and KCNE1. In addition to their ability to modulate the electrophysiological properties of ion channels, it was reported that PUFAs decrease the expression levels of K_v1.5 channels.[19] Our data showed that long-term administration (48 h) of PUFAs decreased the protein levels of K_v7.1, but not those of KCNE1, in a concentration-dependent manner. It was established that internalization and stability of K_v7.1 is regulated through ubiquitinylation by Nedd4–2 and posterior degradation in the proteasome.[27] The decreased K_v7.1 expression was prevented by a proteasome inhibitor, suggesting that PUFAs promote K_v7.1 degradation via proteasome. Secondly, targeting of K_v7.1/KCNE1 to lipid rafts during chronic administration of PUFAs was analysed. K_v7.1 channels partially target lipid rafts in ventricular myocytes,[28] whereas KCNE1 localizes in low-buoyancy fractions only in association with K_v7.1.[22] Our results demonstrated that

chronic administration of EPA and DHA delocalized K_v7.1 in the cell membrane. This suggests an indirect effect of chronic application PUFAs on channel properties, through a change in its membrane environment.

It has been proposed that lipid rafts form signalling platforms in which ion channels, regulatory subunits, scaffolding proteins, kinases, and other modulatory proteins interact.[36] Differential targeting of K_v7.1 and KCNE1 subunits to lipid rafts might represent a critical mechanism of spatial regulation of channel properties as described for other ion channels.[29,37] Correlated changes in K_v7.1 and KCNE1 location in specific microdomains and in their functional properties were shown in the third set of experiments: lipid raft disruption, triggered by MβCD, was followed by measurements of K_v7.1/KCNE1 current, before and after acute application of EPA. Lipid raft disruption (via membrane cholesterol depletion) increased the current magnitude of K_v7.1/KCNE1 at potentials positive to +30 mV and shifted the activation curve towards positive potentials. This observation emphasizes the impact of membrane environment on channel properties. Most importantly, in these conditions, acute EPA application produced similar effects than those observed in non-cholesterol-depleted cells. This suggests a direct effect of EPA during acute application.

Chronic EPA shifts the activation curve to positive potentials, similar to that observed after MβCD treatment. Hence, at least some of the electrophysiological changes on K_v7.1/KCNE1 current after the chronic treatment with EPA might be due to a different location of both subunits on the plasma membrane microdomains. The precise mechanism involved in lipid raft modulation of channel activity is not fully understood. Three different explanations have been proposed: (i) by affecting the biophysical properties of the membrane. Lipid rafts represent thicker and less fluid parts of the membrane than non-raft domains. Changes in the membrane fluidity may modulate ion channel activity. Indeed, it has been described that TRPM8 channel agonists, such as menthol, geraniol, and monoterpenes, enhance the membrane fluidity and increase the ion current.[29] Conversely, increasing the order of the membrane after enrichment with cholesterol inhibits K_v11.1 and K_v7 currents.[38] We can speculate that the location of K_v7.1 and KCNE1 subunits in a thicker and less deformable membrane will increase the energy required for the transition from the closed to the open state. (ii) By specific lipid–protein interactions occurring at raft microdomains. A growing number of studies have demonstrated that cholesterol and PIP_2, major components of the lipid rafts, modulate the activity of several ion channels.[39–41] Moreover, it has been recently reported the binding site for fatty acids in the cavity of the prokaryotic potassium channel KcsA.[42] (iii) By protein–protein interactions. Lipid rafts are enriched in multiple signalling molecules such as G-proteins, protein kinases, phosphatases, etc. It was reported that inhibition of K_v7 channels by cholesterol involves the activity of PKC.[43] Following this reasoning, an impaired interaction between K_v7.1/KCNE1 with adjacent proteins could have an effect on the current magnitude. These three mechanisms are not mutually exclusive.

Overall, our results suggest that PUFAs act on Kv7.1/KCNE1 channels following two mechanisms: (i) a direct one on the channel protein and (ii) an indirect one through rupturing lipid rafts.

4.3 Clinical implications

Acutely applied, PUFAs increased I_{Ks} magnitude by enhancing channel's propensity to remain open. This increased probability of I_{Ks} current remaining open under voltage clamp protocols led to increased I_{Ks} during AP. The reduction of APD was quite small (12 ms for EPA), given the limited role that I_{Ks} has in modulating repolarization under basal physiological conditions. This AP modification *per se* is not likely to have pro- or antiarrhythmic significance. But, the I_{Ks} gain of function could play a relevant role when all the contrasting PUFA effects on cardiac electrophysiology are taken into account. Because such effects are not completely characterized in a quantitative way, we only considered, as a proof of concept, a concomitant I_{Kr} reduction. In this condition, acute PUFA exposure seems to play a significant protective role, by partially compensating the AP prolongation.

On the other hand, the AP simulations also show that chronic exposure to PUFAs does not affect APD_{90}, so that the protective role seems lost in this condition. On the contrary, under extreme I_{Kr} inhibition conditions (80% block), EPA exacerbates the lengthening of the APD. This is a potentially important issue, since it was demonstrated that PUFAs decrease I_{Kr} by 75%.[18]

PUFA antiarrhythmic effects are controversial and some of the reasons contributing to this variability might be the following. First, at the cellular level, PUFAs modify ion channel function through different mechanisms: (i) directly through specific interactions, (ii) modifying the ion channel levels of expression, and (iii) indirectly after rupturing lipid rafts. Secondly, at the population level, different ways of administration and different doses could influence the variability observed in many clinical trials. Other parameters, such as life style or the total amount of fat ingestion, would also modify the risk of cardiovascular events. Finally and more important, it has been demonstrated that PUFA antiarrhythmic potential depends on the subjacent arrhythmogenic mechanism, being antiarrhythmic in triggered activity-induced arrhythmias but deleterious in re-entry arrhythmias.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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