Polyunsaturated fatty acids inhibit K\textsubscript{v}1.4 by interacting with positively charged extracellular pore residues

N. E. Farag,\textsuperscript{1,*} D. Jeong,\textsuperscript{2,*} T. Claydon,\textsuperscript{2,*} J. Warwicker,\textsuperscript{3,*} and M. R. Boyett\textsuperscript{1,*}

\textsuperscript{1}Cardiovascular Medicine, School of Medicine, University of Manchester, Core Technology Facility, Manchester, United Kingdom; \textsuperscript{2}Department of Biomedical Physiology and Kinesiology, Simon Fraser University, Burnaby, British Columbia, Canada; and \textsuperscript{3}Manchester Institute of Biotechnology, The University of Manchester, Manchester, United Kingdom

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Farag NE, Jeong D, Claydon T, Warwicker J, Boyett MR. Polyunsaturated fatty acids inhibit K\textsubscript{v}1.4 by interacting with positively charged extracellular pore residues. Am J Physiol Cell Physiol 311: C255–C268, 2016. First published June 8, 2016; doi:10.1152/ajpcell.00277.2015.—Polyunsaturated fatty acids (PUFAs) modulate voltage-gated K\textsuperscript{+} channel inactivation by an unknown site and mechanism. The effects of ω-6 and ω-3 PUFAs were investigated on the heterologously expressed K\textsubscript{v}1.4 channel. PUFAs inhibited wild-type K\textsubscript{v}1.4 during repetitive pulsing as a result of slowing of recovery from inactivation. In a mutant K\textsubscript{v}1.4 channel lacking N-type inactivation, PUFAs reversibly enhanced C-type inactivation (κ\textsubscript{C}, 15–43 μM). C-type inactivation was affected by extracellular H\textsuperscript{+} and K\textsuperscript{+} as well as PUFAs and there was an interaction among the three: the effect of PUFAs was reversed during acidosis and abolished on raising K\textsuperscript{+}. Replacement of two positively charged residues in the extracellular pore (HS08 and KS32) abolished the effects of the PUFAs (and extracellular H\textsuperscript{+} and K\textsuperscript{+}) on C-type inactivation but had no effect on the lipoelectric modulation of voltage sensor activation, suggesting two separable interaction sites/mechanisms of action of PUFAs. Charge calculations suggest that the acidic head group of the PUFAs raises the pK\textsubscript{a} of HS08 and this reduces the K\textsuperscript{+} occupancy of the selectivity filter, stabilizing the C-type inactivated state.

Polyunsaturated fatty acids (PUFAs) play an important physiological and pathophysiological role in both the brain and heart. The ω-6 PUFA, arachidonic acid (20:4n6), and two major ω-3 PUFAs, eicosapentaenoic acid (20:5n3, EPA) and docosahexaenoic acid (22:6n3, DHA), are crucial components of neural membranes. ω-6 And ω-3 PUFAs are needed for brain growth and functional development during fetal life and infancy (15) and epidemiological data indicate that low ω-3 PUFA intake is a risk factor for Alzheimer’s disease (10). There is evidence that dietary ω-3 PUFAs, abundant in marine organisms, may reduce the development of cardiac arrhythmias and the mortality rate following coronary heart disease in humans (27) and in animal models (4). The concentration of unbound arachidonic acid in normal human serum is small (0.05–0.5 μM), but the concentration is raised in various diseases including diabetes and leukemia (35). In addition, in the brain and heart, the concentration of arachidonic acid rises greatly in ischemia and after seizures (35). For example, in the heart, it is known that myocardial ischemia can result in hydrolysis of phospholipids and release of arachidonic acid from the cell membrane by the activity of phospholipase A\textsubscript{2} (3). In the ischemic heart, arachidonic acid also could accumulate as a result of hormonal activation or the failure of fatty acid oxidation (3). The concentration of ω-3 PUFAs in plasma of humans consuming a regular Western diet ranges from 8 to 12 μM, but consuming moderate to high fish intake for several months can raise plasma ω-3 PUFA concentrations to 200–400 μM (42).

The PUFAs inhibit the transient outward K\textsuperscript{+} current both in the brain and the heart (see Discussion) and the aim of this study was to investigate the mechanism of action of ω-6 and ω-3 PUFAs on K\textsubscript{v}1.4. A number of previous studies have investigated the effects of PUFAs on K\textsubscript{v} channels (21, 23, 34, 40, 43, 48, 49); however, the site and mechanism of action of PUFAs are uncertain. DHA inhibits K\textsubscript{v}1.2 channels, an effect that has been ascribed to external block (40), while linoleic acid modulates K\textsubscript{v}2.1 from the extracellular side (34). Likewise, the inhibitory effects of α-linolenic acid, AA, DHA, and linoleic acid on K\textsubscript{v}1.5 were shown to be externally mediated (21, 23, 34). On the other hand, anandamide blocked K\textsubscript{v}1.5 channels significantly faster when applied intracellularly and is competed away for TEA, suggesting intracellular pore block (36). In contrast to this PUFAs-induced inhibition, lower concentrations of PUFAs increased peak current amplitude in K\textsubscript{v}1.1 (17) and K\textsubscript{v}1.5 (23). Enhanced activation has also been described in K\textsubscript{v}1.2 (40) with DHA, K\textsubscript{v}1.1 with AA and DHA (19), and K\textsubscript{v}1.5 and K\textsubscript{v}2.1 with linoleic acid (34). More recent studies in Shaker potassium channels show that PUFAs induce a leftward shift of the voltage dependence of activation by
several millivolts (7). This ‘lipoelectric’ effect stabilizes the open state of Kv channels and was recently shown to rescue wild-type like function in an arrhythmia-inducing mutant Kv7.1 channel in which the open-state of the channel was destabilized by the mutation (29). The underlying mechanism is the best characterized action of PUFAs in Kv channels and has been shown to be due to electrostatic interactions of the PUFA head-group with the voltage sensor of the channel during its final activation transition (6, 7).

Oliver et al. (39) described the conversion of non-A-type channels to rapidly inactivating channels in response to externally applied PUFAs. The authors demonstrated that inactivation was dramatically enhanced by PUFAs and that the effect was mediated by a PUFA-induced collapse of the outer mouth of the pore that induced stabilization of the C-type inactivated state and was not mediated by intracellular block of the pore. Despite characterization of the effects of PUFAs on Kc channels, neither the site of action of PUFAs nor the mechanism by which PUFAs stabilize the collapsed, inactivated, configuration of the outer pore is known. Here, we studied the action of the ω-6 PUFA, arachidonic acid, and the ω-3 PUFAs, EPA, and DHA on Kv1.4 channel function and demonstrate that the PUFA-induced inactivation is mediated by an outer pore histidine residue, whose charge modification by the PUFA head-group destabilizes K+ occupancy within the outer pore and that this stabilizes the inactivated state. We show that this underlies what was observed as an apparent extracellular block of Kc channels. Moreover, our mutagenesis dissected the effect of PUFAs from the lipoelectric effect and demonstrates that there are at least two separable effects of external PUFAs in Kc channels.

MATERIALS AND METHODS

Molecular biology. Experiments were carried out on: wild-type K1.4 (rat); a truncated version of K1.4 (ferret), K1.4 Δ2–146, lacking rapid N-type inactivation; and two mutants of the truncated channel, K1.4 Δ2–146 H508C and K1.4 Δ2–146 K532C (13). Since rat and ferret K1.4 channel isoforms exhibit very high sequence homology (only 14 of the 654 amino acids differ; 1 divergence in the transmembrane core of the channel and 13, mostly conservative, differences in the cytoplasmic domains), and both display rapid N-type inactivation and slower C-type inactivation, our interpretations of data from either clone also likely hold for the other. In all cases, vectors (pBluescript SKII+ for rat K1.4 and pBluescript SK+ for ferret K1.4) containing the cDNA sequences were linearized with a restriction endonuclease (Eco RI for rat K1.4 and Asp 718 for ferret K1.4) and cRNA was prepared from these templates with either T7 (rat K1.4) or T3 (ferret K1.4) RNA polymerase (Stratagene). Transcribed RNA was diluted in diethyl pyrocarbonate (DEPC)-treated water to a final concentration of 50 ng/µl (rat K1.4) or 100 ng/µl (ferret K1.4).

Electrophysiology. Xenopus laevis frogs were terminally anaesthetized by immersion in tricaine methanesulphonate (2 mg/ml, Sigma) in accordance with the Home Office Animals (Scientific Procedures) Act of 1986. Simon Fraser University Animal Care Committee and Canadian Council on Animal Care have approved the outlined procedure. Stage V–VI oocytes were isolated and then defolliculated using a combination of collagenase treatment (1 h in 1 mg/ml collagenase type 1A; Sigma) and manual defolliculation. Defolliculated oocytes were incubated in Barth’s medium at 19°C for 2–24 h before injection. Barth’s medium contained the following (in mM): 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.82 MgSO4, 0.33 Ca(NO3)2, 0.41 CaCl2, 5 HEPES, 1.25 sodium pyruvate, 0.1 mg/ml neomycin (Sigma), and 100 units/0.1 mg/ml penicillin/streptomycin mix (Sigma); titrated to pH 7.4 using NaOH. Oocytes were injected with 50 nl of cRNA encoding rat K1.4 (2.5 ng) or ferret K1.4 (5 ng) using a Drummond digital microdispenser (Broomall). In control experiments, oocytes were injected with 50 nl of DEPC-treated water; in these cases, currents were negligible compared with currents in cells injected with cRNA. After injection, oocytes were incubated in Barth’s medium at 19°C for a further 16–48 h. During recording, oocytes were perfused with ND96 solution (in mM: 96 NaCl, 3 KCl, 1 MgCl2, 2 CaCl2, and 5 HEPES; titrated to pH 7.4 using NaOH) at a flow rate of 0.5 ml/min. Experiments were performed at room temperature (20–22°C). Currents were recorded using the two-electrode voltage-clamp technique. Microelectrodes with a resistance of 0.6–3 MΩ (tip diameter, 1–5 µm) when filled with 3 M KCl were used. Membrane currents were recorded using a GeneClamp 500 (Axon Instruments) with computer-driven voltage protocols (Clampware software and Digidata 1200 interface, Axon Instruments). To construct current-voltage relationships for the wild-type Kc,1.4 channel, currents were recorded during 200-ms pulses to potentials between −80 and +90 mV from a holding potential of −80 mV. The pulse frequency was 0.5 Hz (corresponding to a pulse interval of 2 s). To measure recovery of the wild-type K1.4 channel from inactivation, a conditioning-test pulse protocol was used. Currents were recorded during a 200-ms test pulse to +40 mV at different test intervals following a 200-ms conditioning pulse to +40 mV (holding potential, −80 mV). The conditioning pulse frequency was 0.067 Hz (pulse interval, 15 s). In experiments on the mutant K1.4 Δ2–146 channel, currents were recorded during pulses of varying stated duration to +60 mV from a holding potential of −80 mV at a frequency of 0.017 Hz (pulse interval, 60 s). In all cases, quantification of inactivation was obtained as the degree of current decay at 4 s compared with the peak current. Conductance-voltage (G−V) relationships describing activation of K1.4 Δ2–146 and K1.4 Δ2–146 H508C mutant channels were determined from peak tail currents recorded during a −60-mV test voltage step that followed 90-ms depolarizing pulses ranging from −80 to +60 mV (in 10-mV increments) from a holding potential of −80 mV. Arachidonic acid (sodium salt; Sigma) was stored at −20°C as a 3 mM stock concentration in H2O. EPA and DHA (Sigma) were stored at −20°C as a 3 mM stock concentration in 100% ethanol. The PUFAs were diluted in ND96 solution to obtain the desired concentration. The final concentration of ethanol was always <1%. Control experiments showed that 1% ethanol had no effect on K1.4. The PUFAs were diluted in ND96 solution immediately before an experiment; the PUFA-containing solution was made up fresh every 3 h and was protected from light to minimize oxidation of the PUFAs. In experiments in which the pH varied, the ND96 solution was adjusted to the desired pH using NaOH. In experiments in which the K+ concentration was raised, the KCl concentration of the ND96 solution was raised and the NaCl concentration was concomitantly reduced. The KCl and NaCl concentrations used were as follows: 3 and 96 mM (normal), 50 and 49 mM, 100 and 0 mM, and 200 and 0 mM (note that the 200 mM K+ solution was hyperosmotic). Oocytes were perfused with an altered solution for 10 min before recording. Data analysis was performed using pCLAMP software (Axon Instruments), SigmaPlot (SPSS Science), and SigmaStat (Jandel Scientific Software). Data are shown as means ± SE (number of oocytes).

Structural modeling. A comparative model had been constructed previously (31) for the pore domain (S5–S6) of Kv1.4 based on the crystal structure for the equivalent part of rat K1.2 (Protein Data Bank id 2a79) (32). The pore domain was placed at the centre of a cylindrical slab of low dielectric atoms, representing the membrane. Binding of arachidonic acid was modeled using the program SwissPdbViewer (18), with the hydrophobic chain lying approximately along the transmembrane axis, and presented to the nonpolar exterior of the pore domain, without overlapping the additional domains (S1–S4) present in the voltage-gated K+ channels (32). The acidic head group was protruding from the modeled membrane.
To assess the relative charge interactions among the arachidonic acid head group, a K⁺ ion at the entrance to the selectivity filter, and H508, a dipole was balanced between the histidine residue and the S₁ binding site for K⁺ in the selectivity filter. The pattern of potential from this dipole effectively shows the division of neighboring regions into those that interact more strongly with either of these two sites. The charge for these calculations was –1e on H508 and +1e at the selectivity filter, setting up the dipole rather than reflecting physiological charge. Dipole potential was computed using the Finite Difference Poisson-Boltzmann method (49), with relative dielectrics of 4 for protein and membrane and 78.4 for solvent, at an ionic strength of 0.15 M.

RESULTS

PUFAs inhibit Kv1.4 by slowing recovery from inactivation. Wild-type Kv1.4 current was recorded during 200-ms pulses from a holding potential of ~80 mV to potentials up to +90 mV at a pulse frequency of 0.5 Hz. Figure 1 shows that wild-type Kv1.4 current was inhibited by 50 μM arachidonic acid, 15 μM EPA, and 50 μM DHA during repetitive pulsing. Figure 1A shows the effect of arachidonic acid, EPA, and DHA on current at +90 mV and Fig. 1B shows the effect on the current-voltage relationship. The PUFAs reduced wild-type Kv1.4 current at most potentials (Fig. 1B). Extracellular acidosis has a similar action on wild-type Kv1.4 current during repetitive pulsing and it is the result of a slowing of recovery from inactivation (12). A conditioning-test pulse protocol was used to measure the recovery of wild-type Kv1.4 from inactivation at ~80 mV. Figure 2A shows superimposed currents at different test intervals (between the conditioning and test pulses) under control conditions and in the presence of 50 μM arachidonic acid or DHA or 15 μM EPA. Figure 2B shows the time course of recovery from inactivation. Kv1.4 current during the test pulse (measured as the difference between peak current and the current at the end of the pulse) is expressed as a percentage of the current during the conditioning pulse and plotted against the test interval. Figure 2B shows that recovery from inactivation was slowed by arachidonic acid, EPA, and DHA. The recovery was fitted by a double exponential function; the slower time constant was increased to 7.2 ± 1.6 s in the presence of 50 μM arachidonic acid (from 2.1 ± 0.1 s under control conditions; paired t-test, P < 0.01) and 3.5 ± 0.4 s in the presence of 50 μM DHA (from 1.8 ± 0.2 s under control conditions; paired t-test, P < 0.01). Recovery from inactivation in the presence of 15 μM EPA showed a similar trend, the time course was 4.11 ± 1.4 s in the presence of EPA and 2.4 ± 0.9 s under control conditions, but this did not reach statistical significance. Inspection of Fig. 2B suggests that the slowing of recovery from inactivation can explain the inhibition of wild-type Kv1.4 current during repetitive pulsing at a frequency of 0.5 Hz (pulse interval, 2 s; Fig. 1). Indeed this is demonstrated in Fig. 2, C and D, which shows that wild-type Kv1.4 current is no longer reduced by 50 μM arachidonic acid when repetitive pulsing was slowed to a frequency of 0.017 Hz to allow for complete recovery of channels from N-type inactivation between voltage steps.

Effect of PUFAs on C-type inactivation. Although inactivation of wild-type Kv1.4 channels is dominated by rapid entry into the N-type inactivated state, recovery from inactivation is determined by the recovery from C-type inactivation (41). Therefore, it is possible that the PUFAs affect C-type inactivation. The effect of PUFAs on C-type inactivation was investigated using a deletion mutant, Kv1.4 Δ2–146, which lacks N-type inactivation (41). Kv1.4 Δ2–146 current was recorded during 7.5 s pulses to +60 mV (pulse frequency, 0.017 Hz)

![Figure 1](http://ajpcell.physiology.org/)
from a holding potential of \(-80\) mV. During the pulse, C-type inactivation was observed - it was slower than N-type inactivation (compare Figs. 1 and 3). Figure 3A shows \(\text{Kv1.4}_{\Delta 2-146}\) current recorded under control conditions and during the application of arachidonic acid. At this slow rate of pulsing, the application of arachidonic acid did not reduce peak current but accelerated the rate of entry of channels into the C-type inactivated state. The effect of arachidonic acid appeared to occur maximally upon the first voltage pulse in the presence of the PUFA and did not alter with prolonged exposure. These data are consistent with those collected in N-type inactivating wild-type channels suggesting that arachidonic acid does not block the channel but rather stabilizes the C-type inactivated state. This is further supported by the data shown in Fig. 3, B and C, which demonstrate the effects of arachidonic acid on \(\text{Kv1.4}_{\Delta 2-146}\) current decay during voltage pulses of duration that varied from 7.5 to 30 s. These data show that current decay approximates a steady-state value by the end of a 30-s long pulse to \(+60\) mV that represents the equilibrium between open and inactivated states. Arachidonic acid reduced the current level indicative of an induced stabilization of the C-type inactivated state. This bias towards the C-type inactivated state could be measured with similar accuracy at numerous time points throughout the current decay (Fig. 3C), and therefore, to simplify data acquisition and analysis, all future quantification of inactivation is presented as the percentage current decay at 4 s.

Figure 3, D and E, shows the effect of different concentrations of arachidonic acid, EPA, or DHA on C-type inactivation in \(\text{Kv1.4}_{\Delta 2-146}\) channels. Arachidonic acid, EPA, and DHA stabilized the C-type inactivated state in a concentration-dependent manner. This is highlighted by the dose-response curves in Fig. 3E in which the percentage inactivation at 4 s is plotted against the concentration of the PUFAs. The \(K_d\) for the effect of arachidonic acid, EPA, and DHA on C-type inactivation was 43, 15, and 18 \(\mu\text{M}\), respectively.

Interaction among the effects of extracellular PUFAs, \(H^+\), and \(K^+\) on \(\text{Kv1.4}\). C-type inactivation of \(\text{Kv1.4}\) is also known to be affected by extracellular \(H^+\) and \(K^+\) (12, 13, 28). Figure 4A shows \(\text{Kv1.4}_{\Delta 2-146}\) current in different extracellular \(K^+\) concentrations (from 3 to 200 mM) and at different extracellular pH values (from 6.5 to 8.5). At each extracellular \(K^+\) concentration, decreasing the pH value can be seen to stabilize the C-type inactivated state. On the other hand, raising the extracellular \(K^+\) concentration can be seen to destabilize the C-type inactivated state. Furthermore, Figure 4A shows that there was an interaction between the effects of extracellular \(K^+\) and extracellular pH, because the effect of extracellular pH was...
less at higher extracellular K\(^+\) concentrations; conversely, the effect of extracellular K\(^+\) was greater at lower pH values. This is confirmed by the titration curves in Fig. 4B: the percentage inactivation at 4 s is plotted against the concentration of extracellular H\(^+\). The titration curve for K\(_{\alpha1.4}\) Δ2–146 is shifted to the right at higher extracellular K\(^+\) concentrations. In Fig. 4C, the pK\(_a\) (the pH value at which the pH effect is half maximal) is plotted against the extracellular K\(^+\) concentration. The pK\(_a\) decreased as the extracellular K\(^+\) concentration was raised reflecting a 30-fold increase in the K\(_d\) of proton binding in response to increasing external K\(^+\) from 3 to 200 mM.

The effect of a decrease in extracellular pH or extracellular K\(^+\) on C-type inactivation? Figure 5A shows the effect of 30 μM arachidonic acid, EPA and DHA on K\(_{\alpha1.4}\) Δ2–146 current at pH 7.4. In each case, the presence of the PUFA reduced the effect of acidic pH on C-type inactivation. In Fig. 5B, titration curves for K\(_{\alpha1.4}\) Δ2–146 are shown in the absence and presence of 30 μM arachidonic acid. They show that, in the presence of arachidonic acid, the titration curve was flattened, i.e., the pH dependence of the channel was markedly reduced. The titration curves in Fig. 5B also suggest that arachidonic acid caused a destabilization of the C-type inactivated state at acidic pH. Although titration curves in the presence of EPA and DHA are not shown, mean data in Fig. 5B confirm that EPA and DHA also destabilized the C-type inactivated state at pH 6.0.

Figure 6A shows the effect of 30 μM arachidonic acid, EPA, and DHA on K\(_{\alpha1.4}\) Δ2–146 current in 3 and 100 mM extra-
cellular K⁺. Whereas in 3 mM extracellular K⁺ the PUFAs enhanced C-type inactivation, in 100 mM K⁺ they had little effect (Fig. 6A). This is confirmed by Fig. 6B, which shows the percentage inactivation at 4 s in the absence and presence of the PUFAs in 3 and 100 mM extracellular K⁺. The results shown in Figs. 5 and 6 demonstrate that there is an interaction among the effects of extracellular PUFAs, H⁺, and K⁺ on C-type inactivation.

**Involvement of two positively charged pore residues (H508 and K532).** We have previously shown that H508 and K532 mediate the acidosis-induced enhancement of C-type inactivation of Kv1.4 (13). Because there is an interaction among the effects of extracellular PUFAs, H⁺, and K⁺ on C-type inactivation, all three may have a common site of action. We, therefore, investigated the effect of PUFAs on Kv1.42–146 in which H508 or K532 was substituted by a cysteine residue. Figure 7 shows the effect of 30 μM arachidonic acid, EPA, and DHA on the mutated forms of Kv1.42–146 (typical currents and mean percentage inactivation at 4 s are shown). However, whereas 30 μM arachidonic acid, EPA, and DHA had no effect on both Kv1.4 Δ2–146 H508C and Kv1.4 Δ2–146 K532C (Fig. 7), Higher concentrations of arachidonic acid (70 μM) failed to induce a notable change in the rate of or steady-state C-type inactivation in Kv1.42–146 H508C channels (data not shown), suggesting that the effect of PUFAs on C-type inactivation is abolished with the mutations.

**Two separable mechanisms of action of arachidonic acid.** Interestingly, although the H508C mutation abolished the effect of arachidonic acid on C-type inactivation, the mutation had no impact on the effect of arachidonic acid on the voltage dependence of activation. Figure 8A shows that arachidonic acid induces a hyperpolarizing shift in the voltage dependence of Kv1.42–146 activation in a manner that is similar to that described previously for the action of PUFAs in Shaker, Kv1.2, Kv1.5, Kv2.1, Kv7.1, and Kv11.1 channels (7, 19, 30, 34, 40). Kv1.42–146 currents recorded in response to 90-ms step depolarizations applied from −80 to +60 mV are shown along with relative conductance-voltage relationships plotted from peak tail currents recorded at −60 mV and fitted using the Boltzmann equation. Application of arachidonic acid induced an $-8.8 \pm 1.5$ mV ($n = 11$) shift in the $V_{1/2}$ of activation. Such modulation of the voltage dependence of Kv channel activation by PUFAs has been best characterized in Shaker channels and has been described as a lipoelectric effect due to an electro-
Further examination of the effects of arachidonic acid on Kv1.4/H90022–146 H508C mutant channels (Fig. 8B) reveals that the lipoelectric effect of arachidonic acid is preserved in H508C channels despite the abolition of the effect on C-type inactivation. In Kv1.4/H90042–146 H508C channels, arachidonic acid induced an $-11.6 \pm 1.0$ mV shift ($n = 5$; $t$-test $P > 0.1$ compared with the shift induced in Kv1.4 Δ2–146 channels) in the $V_{1/2}$ of activation. These data suggest two effects of arachidonic acid in Kv1.4 channels that are separable by selective abolition of the effects on C-type inactivation by the H508C outer pore mutation, which does not alter the PUFA-induced lipoelectric effect.

Structural modeling. Figure 9A shows a section through the modeled Kv1.4 pore domain (S5–S6) used in our charge calculations: only two of the four subunits are shown for clarity and the positions of H508 and K532 are shown. This was brought together with an arachidonic acid molecule in a cylindrical membrane slab. Arachidonic acid (shown in Fig. 9A, left), like other PUFAs, consists of a hydrophobic tail and a negatively charged (i.e., acidic) head group (shown in red in Fig. 9A, left). Arachidonic acid is modeled in the membrane.
with the negatively charged head group protruding (Fig. 9B). The calculated dipolar potential from H508 (shown in red in Fig. 9B; see MATERIALS AND METHODS) suggests that arachidonic acid could interact more strongly with the charge at H508 than with the charge at the selectivity filter. This suggests only a relatively small direct interaction between the negatively charged arachidonic acid head group and a K\(^{+}\)/H11001 ion in the selectivity filter. Figure 9B suggests instead that there is an indirect interaction: the negative charge of the arachidonic acid head group is expected to stabilize the protonated form of H508 (i.e., raise its pK\(_a\)), which in turn is expected to destabilize K\(^{+}\)/H11001 ions in the selectivity filter, leading to the observed stabilization of the C-type inactivated state. In the absence of H508, there would be a lesser effect or no effect of PUFAs, as observed in Fig. 7A.

**DISCUSSION**

This study has shown that K\(^{+}\) current flow through the K\(_v1.4\) channel is inhibited by PUFAs during repetitive pulsing and that this is the result of a slowing of recovery of K\(_v1.4\) from inactivation, which in turn arises from a stabilization of channels in the C-type inactivated state. The effect of PUFAs is similar to that of extracellular acidosis or a decrease of extracellular K\(^{+}\)/H11001 and indeed there is an interaction among the three effects. The effect of PUFAs can be abolished by mutation of two positively charged residues in the extracellular pore region of the channel, as can the effects of extracellular H\(^{+}\) and K\(^{+}\). A model has been put forward to explain the action of PUFAs at the molecular level, which suggests that the mechanism by which PUFAs act on C-type inactivation is mediated by outer pore residues and is distinct from the lipoelectric
effect of PUFAs on the voltage dependence of Kv channel activation that is mediated via interactions with the voltage sensor.

**Mechanism of action of PUFAs on C-type inactivation.** Although in no other study has the site of action of PUFAs on C-type inactivation been addressed, inactivation in Kv4 (22, 43) and Kv1.5 channels (23) has been reported to be enhanced by arachidonic acid and DHA. Oliver et al. (39) also showed that arachidonic acid enhanced inactivation in Kv3.1 channels and went on to show that the effects are not mediated via intracellular pore block but rather conformational changes at the selectivity filter. However, in none of these studies was evidence provided for a site and mechanism of action of PUFAs on inactivation.

The data in Fig. 2C and 3A suggest that PUFAs act at an extracellular site outside of the permeation pathway to modify C-type inactivation in Kv1.4 and Kv1.4 Δ2–146 channels. A pore block mechanism is inconsistent with the observation that peak Kv1.4 current is not inhibited by arachidonic acid when the pulse frequency is slowed to allow for complete recovery from inactivation (Fig. 2C). Furthermore, in Kv1.4 Δ2–146 channels, the maximal effect of a given concentration of arachidonic acid can be observed in the first recording following application of the PUFAs (Fig. 3A), suggesting an extracellular site of action and no accumulation of channel block. This is also consistent with the observation that mutation of the outer pore residues, H508 and K532, abolishes the effects of arachidonic acid on C-type inactivation (Fig. 7). Such side-specificity of PUFAs interaction has been reported previously in other Kv channels. DHA inhibits Kv1.2 channels extracellularly (40), and linoleic acid modulates Kv2.1 from the extracellular side (34). Similarly, the inhibitory effects of α-linolenic acid, arachidonic acid, DHA, and linoleic acid on Kv1.5 channels are externally mediated (21, 23, 34). In contrast, the arachidonic acid derivative anandamide inhibits Kv1.5 channels via an intracellular pore block mechanism (36). A non-specific action of PUFAs on Kv1.4 (for example, a detergent effect and a change in membrane fluidity) is unlikely, because the PUFAs-induced inactivation of the channel was reversed by extracellular acidosis (Fig. 5), abolished by raised extracellular

![Fig. 7. Effect of PUFAs on the Kv1.4 Δ2–146 H508C and Kv1.4 Δ2–146 K532C mutant channels. A and B: Kv1.4 Δ2–146 H508C (A) and Kv1.4 Δ2–146 K532C (B) current traces recorded under control conditions and in the presence of 30 μM arachidonic acid (top), 30 μM EPA (middle), and 30 μM DHA (bottom). The currents were recorded during a 15-s voltage pulse to +60 mV from a holding potential of −80 mV. The current traces have been normalised to the peak current. Insets: percent inactivation at 4 s under control conditions and in the presence of the PUFAs. Means ± SE (n = 5) are shown. pH 7.4: extracellular K⁺ concentration: 3 mM.](image-url)
on the voltage dependence of activation. Börjesson et al. (6, 7) characterized the modification of activation voltage dependence by cis ω-3 and ω-6 fatty acids in Shaker channels as a lipoelectric mechanism, where charged PUFAs electrostatically modulate the voltage dependence of activation.

K+ (Fig. 6), and abolished by the mutation of two positively charged extracellular pore residues, H508 and K532 (Fig. 7). An outer pore PUFA interaction site that modulates C-type inactivation in K1.4 channels is therefore a novel finding that is consistent with previous observations of the effects of PUFAs in other channels.

Separable mechanisms of PUFA-induced modulation of K1.4 channel function. There is evidence that PUFAs interact with K+ channels via multiple sites/mechanisms. For example, in addition to inhibiting current, PUFAs have been shown to shift the voltage dependence of activation to more negative potentials in Shaker (7), K+,1.2 (40), K+,1.5 and K+,2.1 (34), K+,7.1 (30), and K+,11.1 (19) channels. Data shown in Fig. 8 demonstrate a similar phenomenon in K1.4 channels. Interestingly, the H508C mutation abolished the effects of arachidonic acid on C-type inactivation without modifying the effect of the voltage dependence of activation. Börjesson et al. (6, 7) characterized the modification of activation voltage dependence by cis ω-3 and ω-6 fatty acids in Shaker channels as a lipoelectric mechanism, where charged PUFAs electrostatically modulate the voltage dependence of activation.

Fig. 8. Lipoelectric effect of arachidonic acid on wild-type K1.4 Δ2–146 and K1.4 Δ2–146 H508C channels. A and B: the voltage dependence of activation of wild-type K1.4 Δ2–146 (A) and K1.4 Δ2–146 H508C channels (B) in the absence (●) and presence of 70 μM arachidonic acid (○). The lipoelectric effect of arachidonic acid is indicated as a hyperpolarizing shift of the voltage dependence of activation. Insets: tonic current traces in the presence of arachidonic acid recorded from short 90-ms depolarizations from a −80-mV holding potential to +60 mV in 10-mV increments, followed by a repolarizing step to −60 mV. Means ± SE (n = 11 and 5, respectively) are shown.

Fig. 9. Modelling the interaction of arachidonic acid with the pore domain of K1.4. A: a spacefilling representation of arachidonic acid, EPA, and DHA alongside 2 subunits of a model (31) of the K1.4 pore domain (S5–S6), drawn as a backbone trace. The other 2 subunits are omitted for ease of viewing. B: a structural model for arachidonic acid interaction that is consistent with the experimental data. Arachidonic acid was modeled with the hydrophobic chain along the transmembrane axis and the acidic head group protruding. The view is into the membrane extracellular face, and shows the distribution of groups radially from the central pore axis, with H508 closer in and the arachidonic acid head group further out. Note that arachidonic acid could be positioned at about this radius, whether the full K1.4 transmembrane channel (S1–S6) or just the pore domain (S5–S6) are included. The pore domain (S5–S6) is drawn as a salmon-colored backbone, within the grey slab of membrane used in the calculations. Red and blue contours show the result of electrostatics calculation with the Finite Difference Poisson Boltzmann method (49), for a dipole balanced between a single H508 site and the S1 K+ conduction axis, supporting a model in which H508 protonation state mediates the effect of arachidonic acid. Swiss-Pdb Viewer (18) aided the preparation of this figure.
PUFA-modulated channels typically interact with the voltage sensor during its final outward transition leading to the channel opening. Recently, Li et al. (30) also demonstrated a similar PUFA modulation and binding site on K,7.1 channels. The data in the present study are the first, to our knowledge, to demonstrate that the lipoelectric effect is observed in K,1.4 channels, and therefore, we chose to focus on the separation of this effect from that on inactivation using a test candidate, arachidonic acid. The similarity in the lipoelectric effect of a range of PUFAs in the above-mentioned channels would suggest that we would see a similar result in K,1.4 channels with DHA and EPA. The separation of effects of arachidonic acid in K,1.4 channels by the HS08C mutation suggests that there are two extracellular arachidonic acid interaction sites, one on the voltage sensor, which mediates the lipoelectric effect, and one in the outer mouth of the pore, which mediates the effect on C-type inactivation. Alternatively, a common interaction site may elicit two separable effects, one of which, that on C-type inactivation, can be disrupted by the HS08C mutation. The modeling data shown in Fig. 9, which suggest that arachidonic acid interacts directly with HS08 to alter its charge and therefore pore occupancy of K+ and consequently C-type inactivation (see below), favors the former possibility. Interestingly, a previous study describing the effect of linoleic acid on K,1.5 and K,2.1 channels also concluded that there are two distinct mechanisms that allow the PUFA to affect both activation and inactivation, although the exact mechanisms were not established (34).

Interaction among the effects of extracellular PUFAs, H+, and K+ and the role of pore charges. The effect of PUFAs on C-type inactivation of K,1.4 was similar to the actions of extracellular H+ and K+ (Figs. 3 and 4). There was an interaction between the effects of extracellular H+ and K+ on the K,1.4 channel: acidosis acted to increase C-type inactivation and this effect was reduced on moving from 3 to 200 mM K+, which by itself acted to decrease C-type inactivation (Fig. 4). There was also an interaction among the effects of extracellular PUFAs, H+, and K+: extracellular acidosis reversed the PUFA-induced enhancement of C-type inactivation of K,1.4, while raised extracellular K+ abolished it (Figs. 5 and 6). An interaction between extracellular H+ and K+ in mediating C-type inactivation is consistent with a previous study from our laboratory on the wild-type K,1.4 channel (14) [see also Li et al. (28)], as well as studies in K,1.3 (45), K,1.4 (12), (13), and K,1.5 (16) channels, which identify outer pore residues, e.g., HS08 and K532 in K,1.4, as pH sensor sites. It is well established that raising extracellular K+ abolishes C-type inactivation by occupying the pore and preventing pore collapse, while acidosis enhances C-type inactivation (via interaction with HS08) decreasing K+ occupancy of the pore and thus enhancing C-type inactivation (1, 13, 31, 41). We, and others, have also shown that raising extracellular K+ abolishes the effect of acidosis by preventing the pore collapse due to inactivation (13, 31). Here, we propose that PUFAs act, like acidosis, via HS08. This conclusion is consistent with the PUFA-induced stabilization of C-type inactivation (Fig. 3) as well as the observation that extracellular K+ abolishes the effect of PUFAs (Fig. 4). Figure 5 shows that there is an interaction between the action of PUFAs and H+, where the effect of PUFAs was reduced in the presence of protons. Borjesson et al. (7) observed a similar pH dependence to the effect of PUFAs on the lipoelectric effect in Shaker channels.

They reasoned that protonation of the PUFA headgroup reduced the effect of the PUFA suggesting an electrostatic mechanism. We propose that protonation of membrane-embedded PUFAs at low pH alters the PUFA interaction with the HS08 charge in a similar way, preventing it from stabilizing the inactivated configuration. Depending on the pH, not all PUFAs will be protonated and some HS08 sites may be free to interact with protons producing a net effect of a partial relief of the PUFA-induced stabilization of inactivation at acidic pH (Fig. 5).

The data in Fig. 7 show that mutation of HS08 or K532 extracellular charges abolished the PUFA-induced enhancement of C-type inactivation. The effect of mutation of HS08 was specific in its abolition of the action of PUFAs on inactivation, without modifying the effect of PUFAs on activation gating, which provides strong evidence for a role of this site in mediating the effects of PUFAs on K,1.4 inactivation. Modeling studies (Fig. 9) suggest that increasing the positive charge at HS08 stabilizes C-type inactivation by increasing repulsive interactions with K+ ions. We propose that mutation of K532 alters inactivation. In previous work, we showed that K532 is an important site for inactivation in K,1.4 channels, not as a titratable site, but rather as a site that tunes the electrostatic profile of the pore that a K+ ion encounters (14, 31). Continuum electrostatic modeling suggested that mutations at K532 alter the electrostatic profile such that K+ occupancy is favored regardless of charge modification at HS08 (31). The data in the present study are consistent with this in that mutation of K532 abolishes the effects of PUFAs on C-type inactivation. These data suggest that mutation of K532 increases K+ occupancy to such an extent that PUFA modification of HS08 charge does not reduce K+ occupancy sufficiently to stabilize C-type inactivation. Our interpretation of these collected data is that HS08 is the proton-sensor and also the PUFA-sensitive residue with protons and PUFAs acting via a common mechanism that involves K532, which is known to be linked to C-type inactivation in K,1.4 and other channels (12, 33).

A possible common mechanism of action of PUFAs. Modelling the effects of extracellular protons on K+ coordination within the K,1.4 outer pore has suggested that the increased positive charge of the protonated HS08 residue electrostatically reduces K+ occupancy of the selectivity filter, thus promoting C-type inactivation (31). K+ occupancy of the selectivity filter is thought to control the constriction of the selectivity filter during C-type inactivation (38), which is dictated by the exit of K+ from the pore (2). As shown in Fig. 9, it is proposed that the hydrophobic tail of PUFAs lies within the membrane and that the negatively charged head group interacts with HS08. The effect of this will be to favor protonation of HS08 (i.e., to increase the pKs of HS08) and, therefore, ultimately C-type inactivation. The experimental data suggest that a PUFA at its Kd must increase the pKs for C-type inactivation from 6.7 (normal value at an extracellular K+ concentration of 3 mM; Fig. 4) to 7.4 (because C-type inactivation is half-maximally increased at pH 7.4 in the presence of a PUFA at its Kd; Fig. 5B).

Calculations of the effect of binding one arachidonic acid molecule per monomer unit of the 2a79 crystal structure, i.e., four arachidonic acid molecules in total, as described in MATERIALS AND METHODS, gives an estimate of a pKs shift of ~0.4 for each HS08 sidechain. This estimate would be affected by conformational change and the location of arachidonic acid
binding to transmembrane regions of the pore domain exterior. Nevertheless, compared with the experimentally observed pK_a change of 0.7 it may indicate, within the confines of our model, that more than one arachidonic acid molecule binds per channel monomer. This hypothesis is consistent with the study of Smirnov and Aaronson (44), who found that a neutral fatty acid has a smaller inhibitory effect on delayed rectifier K^+ current in rat pulmonary arterial myocytes than a negatively charged one.

The modeling data in Fig. 9 could explain why extracellular PUFAs, H^+, and K^+ had similar effects and why there was an interaction among them and why mutation of H508 abolishes the effects. We propose that the positively charged K532 residue electrostatically affects the K^+ occupancy of the selectivity filter: it tonically reduces the K^+ occupancy to a point at which C-type inactivation is sensitive to further changes in K^+ occupancy (31). This explains the effect of neutralization of K532: after its replacement there is an increase in K^+ occupancy and the PUFA-facilitated protonation of H508 no longer decreases K^+ occupancy sufficiently to trigger C-type inactivation. One finding that remains to be explained is why acidosis reverses the effect of PUFAs (Fig. 5). We suggest that at low pH PUFAs no longer affect the channel via an indirect effect on the pK_a of H508, but instead, via a weak direct effect (normally masked by the stronger indirect effect): the negatively charged head group of PUFAs may negate some of the positive charge of H508 (which by itself tends to reduce the K^+ occupancy of the selectivity filter and favor C-type inactivation).

Our data suggest that arachidonic acid stabilizes C-type inactivation by modifying the electrostatic potential at the outer mouth of the pore, which reduces K^+ occupancy. Although the rate and extent of C-type inactivation vary greatly between Kv channels, even those within the same subfamily, we speculate that PUFA modification of the potential profile of the outer pore may be a general mechanism by which PUFAs modulate C-type inactivation in Kv channels. Our data would suggest that channels with an equivalent His residue to H508, such as K1.5, would also demonstrate PUFA-dependent stabilization of C-type inactivation via charge modification of the His as reported here for Kv1.4 channels. Indeed, pH dependence of C-type inactivation has been shown in K1.5 to be mediated by an equivalent His pore residue (46). However, the site of action of PUFAs is likely different in different channels, since this His is not highly conserved. For example, it is absent in K1.2 channels, which display PUFA-dependent inactivation. Furthermore, other PUFA-sensitive channels, such as K2.1 and K3.1, undergo a complex inactivation process that involves C-type inactivation but also pronounced U-type inactivation that has been characterized as inactivation from closed states (25, 26). Our findings of the site of action may not extrapolate to such cases, but we propose that PUFA-induced modification of K^+ occupancy may be a general mechanism of action.

The physiological relevance of modulation of Kv1.4 by PUFAs. Besides the effects of PUFAs on cloned channels, PUFAs have also been shown to affect native outward rectifier K^+ currents: for example, in hippocampal neurons and retinal cells, arachidonic acid enhances inactivation and reduces the amplitude of transient outward K^+ current (9, 24). In the heart, the transient outward K^+ current is generated by Kv1.4, Kv4.2, and Kv4.3 channels and EPA and DHA enhance inactivation and reduce the amplitude of the transient outward K^+ current in rat and ferret cardiac myocytes (5, 50).

Arachidonic acid, EPA, and DHA increased C-type inactivation of Kv1.4 with a K_d of 43, 15, and 18 μM, respectively (Fig. 3). This suggests that Kv1.4 can be modulated by the PUFAs under physiological and pathophysiological conditions: the plasma concentration of ω-3 PUFAs in the human can be up to 200–400 μM (see Introduction). In the heart, because Kv1.4 is one of the channels responsible for I_{to}, an important determinant of the cardiac action potential duration, modulation of Kv1.4 by ω-6 and ω-3 PUFAs may affect the dispersion of repolarization and the propensity for ventricular arrhythmias during myocardial ischemia when the concentration of arachidonic acid is raised (11). However, modulation of Kv1.4 by PUFAs is unlikely to be the only mechanism responsible for any beneficial action of PUFAs on the heart, because the PUFAs affect other targets. If modulation of Kv1.4 by PUFAs is important, as shown in Figs. 5 and 6, the effect of PUFAs is expected to be affected by extracellular pH and K^+. Accumulation of PUFAs, acidosis, and raised extracellular K^+ occur in many pathological conditions. For example, in the heart, during metabolic inhibition, extracellular pH can decrease to 6, extracellular K^+ can increase to 12 mM and PUFAs can be released as a result of phospholipase A_2 activity. The PUFAs, acidosis, and raised extracellular K^+ will each affect Kv1.4, but there will also be an interaction among the effects. The consequences of such pathological conditions are, therefore, complex.

Limitations. Despite the sequence similarity between the ferret and rat Kv1.4 clones (see MATERIALS AND METHODS), the use of different Kv1.4 isoforms to study the effect of PUFAs on C-type and N-type inactivation may limit our interpretations. However, although these studies used slightly different isoforms, the fundamental mechanistic properties of N- and C-type inactivation are conserved in both Kv1.4 channel types. As such, this approach supports our conclusions that PUFAs inhibit Kv1.4 current by stabilizing the C-type inactivated state, which in turn slows the recovery from N-type inactivation. A second potential limitation to this study is that the effects of only single substitutions at H508 and K532 were studied. However, in previous work we have demonstrated, using various modifications at these sites, how H508 might be involved with inactivation using multiple charge altering manipulations. Thus these new data build on a previously described mechanistic role for H508 in the outer pore mouth to provide a working hypothesis for the action of PUFAs on inactivation.

Acknowledgments

Present address of N. E. Farag: Dept. of Physiology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

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