Docosahexaenoic acid alters bilayer elastic properties

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At low micromolar concentrations, polyunsaturated fatty acids (PUFAs) alter the function of many membrane proteins. PUFAs exert their effects on unrelated proteins at similar concentrations, suggesting a common mode of action. Because lipid bilayers serve as the common ''solvent'' for membrane proteins, the common mechanism could be that PUFAs adsorb to the bilayer/solution interface to promote a negative-going change in lipid intrinsic curvature and, like other reversibly adsorbing amphiphiles, increase bilayer elasticity. PUFA adsorption thus would alter the bilayer deformation energy associated with protein conformational changes involving the protein/bilayer boundary, which would alter protein function. To explore the feasibility of such a mechanism, we used gramicidin (gA) analogues of different lengths together with bilayers of different thicknesses to assess whether docosahexaenoic acid (DHA) could exert its effects through a bilayer-mediated mechanism. Indeed, DHA increases gA channel appearance rates and lifetimes and decreases the free energy of channel formation. The appearance rate and lifetime changes increase with increasing channel-bilayer hydrophobic mismatch and are not related to differing DHA bilayer absorption coefficients. DHA thus alters bilayer elastic properties, not just lipid intrinsic curvature; the elasticity changes are important for DHA's bilayer-modifying actions. Oleic acid (OA), which has little effect on membrane protein function, exerts no such effects despite OA's adsorption coefficient being an order of magnitude greater than DHA's. These results suggest that DHA (and other PUFAs) may modulate membrane protein function by bilayer-mediated mechanisms that do not involve specific protein binding but rather changes in bilayer material properties.

bilayer material properties | bilayer stiffness | gramicidin channels | hydrophobic mismatch | polyunsaturated fatty acid

Polyunsaturated fatty acids (PUFAs) modulate a wide variety of biological processes (1–3), and alter the function of a diverse group of unrelated membrane proteins (Table 1, for additional examples, see ref. 4), whereas saturated or monounsaturated fatty acids such as oleic acid (OA) are relatively inert. Among the acute effects of PUFAs is the reversal of the arrhythmias underlying sudden cardiac death in rats (5), dogs (6), and humans (7), most likely due to inhibition of cardiac sodium and L-type calcium channels. The mechanism(s) underlying the reversal remain unclear, but it occurs at the low micromolar PUFA concentrations where PUFAs are general modulators of membrane protein function. Because PUFAs avidly adsorb to biological membranes (8, 9), and the commonality among the proteins in Table 1 is that they are imbedded in lipid bilayers, PUFAs may act through some common, bilayer-mediated mechanism.

Bilayer-dependent regulation of membrane function can occur when membrane proteins undergo conformational changes that involve the protein/bilayer boundary (for example, see ref. 21). Because lipid bilayers are elastic bodies (22) and bilayer-spanning proteins are coupled to the host bilayer through hydrophobic interactions (23), membrane protein conformational changes incur an energetic cost (24–26), the bilayer deformation energy (ΔG^0_{def}), which causes protein function to be modulated by the lipid bilayer (27, 28).

Table 1. Membrane proteins that are modulated by DHA and other PUFAs

Protein	PUFA	Action	Ref.
Cardiac Na^+ channel	AA, EPA, DHA	Inhibit	10
L-type Ca^{2+} channel	ALA, LA, AA, EPA, DHA	Inhibit	11
Kv1.5 channel	AA, DHA	Inhibit	12
HERG channel	AA, DHA	Inhibit	13
TRAAK-1 channel	AA, EPA, DHA	Activate	14
TRPV1	EPA, DHA	Activate	15
nAChR channel	DHA	↑ Desensitization	16
$GABA_a$ channel	DHA	↑ Desensitization	17
GluR6 glutamate receptor	AA, DHA	Inhibit	18
Connexin43 channel	GLA, AA, EPA, DHA	Inhibit	19
Na^+,K^+-ATPase	EPA, DHA	Inhibit	20

AA, arachidonic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid

For a given protein, ΔG^0_{def} varies as a function of the mismatch between the protein's hydrophobic length (l) and the bilayer's hydrophobic thickness (d_0), the intrinsic curvature (c_0) of the bilayer-forming lipids, and the bilayer compression and bending moduli. To a first approximation, ΔG^0_{def} can be expressed as a biquadratic form in the hydrophobic mismatch, $d_0 - l$, and c_0 (28):

$$\Delta G^0_{def} = H_B \cdot (d_0 - l)^2 + H_X \cdot (d_0 - l) \cdot c_0 + H_C \cdot c_0^2, \quad [1]$$

where the coefficients H_B, H_x, and H_c are determined by the protein geometry, the bilayer thickness and elastic moduli (29). The elastic moduli (30–32) and intrinsic curvature (33, 34) may be altered by reversibly adsorbing amphiphiles, which would provide a basis for the acute effects of PUFAs on membrane protein function, although the relative importance of changes in elastic moduli and curvature would need to be established. PUFAs and micelle-forming amphiphiles, for example, have opposite effects on lipid intrinsic curvature (34, 35), yet both shift the steady-state inactivation curve for voltage-dependent sodium channels in the same (hyperpolarizing) direction (35, 36), which would suggest that changes in elastic moduli dominate over changes in curvature.

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Abbreviations: PUFA, polyunsaturated fatty acid; OA, oleic acid; gA, gramicidin; DHA, docosahexaenoic acid.

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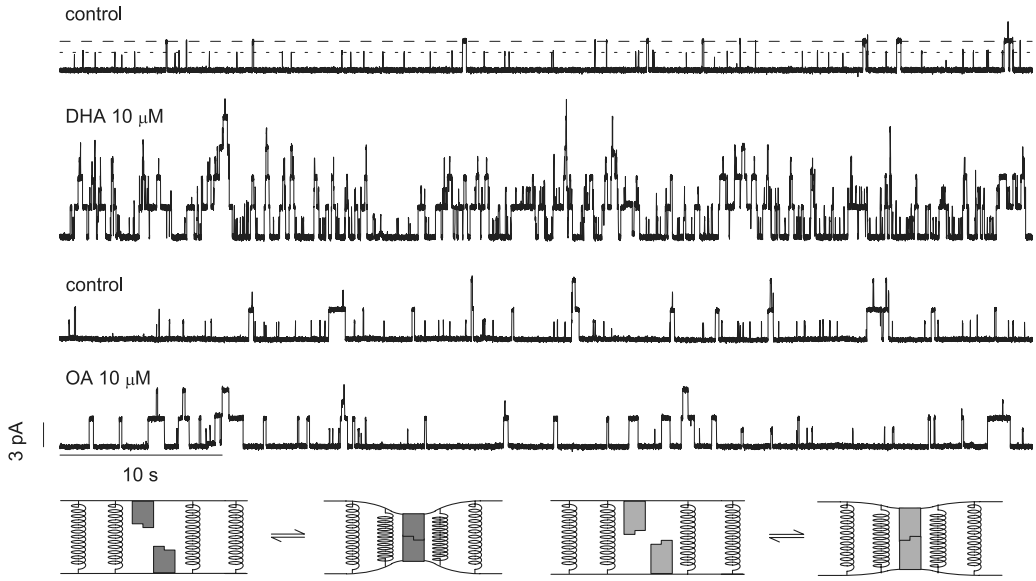


Fig. 1. Effect of OA and DHA on gA channel activity. Current traces before and after addition of 10 μM DHA (top two traces) or OA (bottom two traces) to both sides of a $DC_{18:1}PC$/*n*-decane bilayer containing gA(13) and $AgA^{-}(15)$. (Results from two different experiments.) The interrupted lines denote the current levels for gA(13) (short dash) and $AgA^{-}(15)$ (long dash). 1 M NaCl, 10 mM Hepes, pH 7, $\pm$ 200 mV, 500 Hz. The cartoons at the bottom of the figure illustrate the differences in bilayer deformation with differing hydrophobic mismatch between channel (shaded blocks) and lipid bilayer (represented by springs).

To address these questions we used gramicidin (gA) channels of different lengths to monitor how PUFAs modulate lipid bilayer properties. gA channels are formed by transbilayer association of two monomers (37). When the bilayer's hydrophobic thickness, thickness to match the protein's hydrophobic length, differs from the channel's hydrophobic length, l, the bilayer will adjust its d_0 resulting in a local bilayer deformation with energetic cost $\Delta G^0_{\rm def}$ (cf. Eq. **1**). The bilayer responds by applying a disjoining (restoring) force to the channel dimer:

$$F_{\rm dis} = 2{\cdot}H_{\rm B}{\cdot}(d_0 - l) + H_{\rm X}{\cdot}c_0. \qquad [2]$$

Changes in this disjoining force are reflected as changes in channel lifetime (τ), meaning that gA channels can be used as force transducers (38) to report changes in bilayer elasticity and lipid curvature. It is thus possible to assess whether docosahexaenoic acid (DHA) alters bilayer properties and thus might exert its effects on membrane protein function through an indirect (bilayer-mediated) mechanism.

Results

DHA is a potent modifier of gramicidin channel activity, whereas OA is not (Fig. 1). One can examine the role of channel-bilayer hydrophobic mismatch by comparing the relative changes in lifetimes and appearance rates for the shorter and longer channels (Fig. 1 *Lower*). 10 μM DHA[§], but not 10 μM OA, increases the appearance rates of both gA(13) and $AgA^{-}(15)$ channels with the larger effect on the shorter channels (Figs. 2 and 3). [The enantiomeric gA(13) and $AgA^{-}(15)$ were used to prevent hybrid channel formation, which simplifies the analysis.]

DHA also increases the single-channel current transition amplitudes (i), whereas OA has little or no effect (Fig. 2*A*). The lifetime distributions in the absence or presence of DHA (or OA) can be fit by single exponential distributions (Fig. 2*B*), meaning that DHA modulates the function of an existing channel type, rather than promoting the appearance of a new channel population. (In the absence of gA, neither DHA nor OA caused channel-like activity.)

When DHA was added to only one side of a bilayer, the single-channel currents and lifetimes were similar at both polarities (results not shown), indicating that transmembrane DHA flux (for example, see refs. 39 and 40) caused the DHA mole fraction to be similar in the two leaflets.

As will be important below, DHA is more active in altering the function of the shorter gA(13) channels. This difference is not

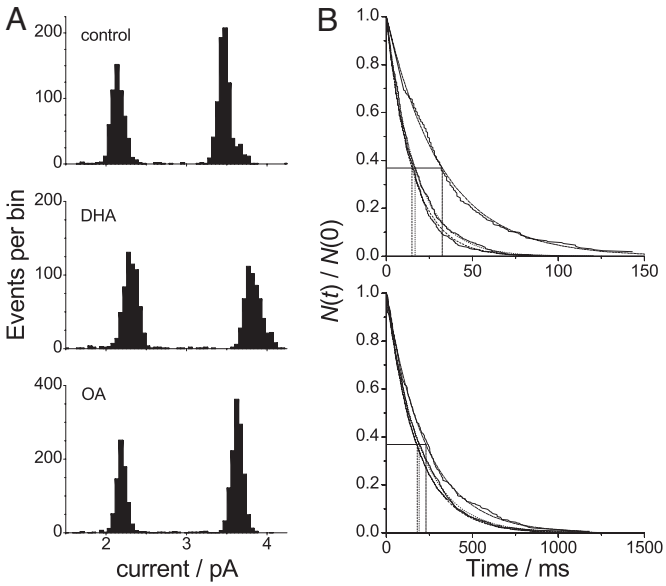


Fig. 2. Effect of OA and DHA on gA single-channel current transitions and lifetimes. (*A*) Current transition amplitude histograms of gA(13) (left peak) and $AgA^{-}(15)$ (right peak) channels in $DC_{18:1}PC$ bilayers in the absence or presence of 10 μM DHA or OA. (*B*) Normalized single-channel survivor histograms for gA(13) (*Upper*) and $AgA^{-}(15)$ (*Lower*) fitted with single exponential distributions; note the 10-fold difference in the scale of the abscissae. The vertical dotted lines indicate the average channel lifetimes of (from left to right) control, 10 μM OA, and 10 μM DHA.

[§]FAs absorb to surfaces, and the nominal FA concentrations (based on the total amount added) are higher than the actual concentrations (see *Discussion*).

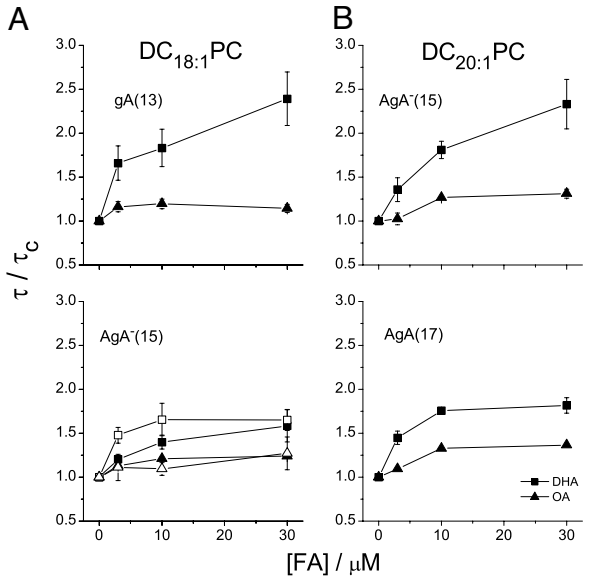


Fig. 3. Concentration dependence of OA's and DHA's effects on channel lifetimes. Changes in the lifetime relative to control are plotted as normalized dose-response curves for DHA (filled square) and OA (filled triangle). (*A*) gA(13) and AgA$^-$(15) channels in $DC_{18:1}PC$ bilayers, as well as AgA$^-$(15) channels in $DC_{18:1}PC$:Chol (1:1) bilayers [DHA (open squares) and OA (open triangles) in *Lower*]; (*B*) AgA$^-$(15) and AgA(17) channels in $DC_{20:1}PC$ bilayers.

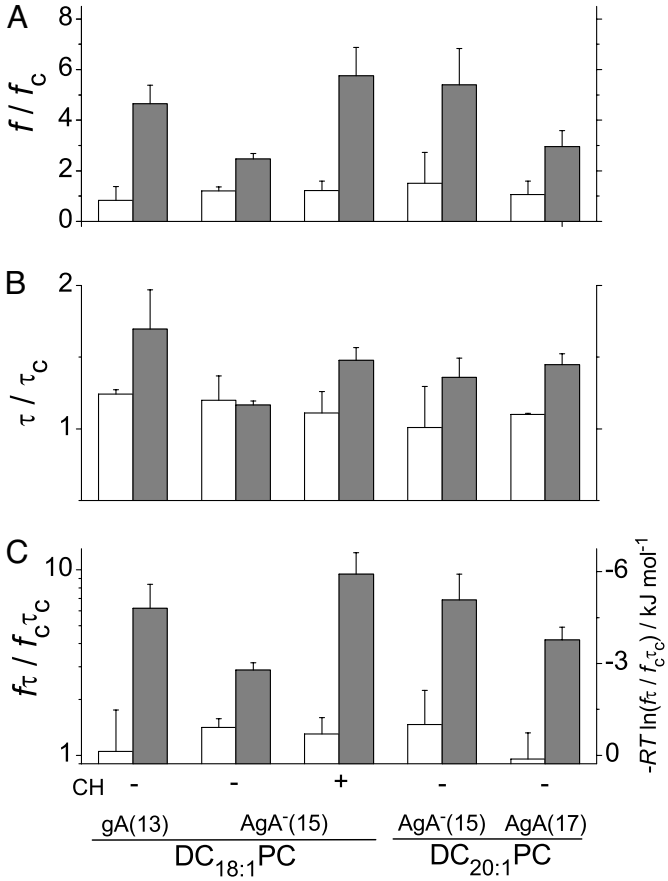


Fig. 4. Changes in kinetics and energetics of channel formation. (*A*) The relative increase in channel appearance rate (f) induced by 3 μM OA (white) or DHA (gray) in $DC_{18:1}PC$, $DC_{18:1}PC$:Chol (1:1) and $DC_{20:1}PC$ bilayers. (*B*) Corresponding changes in gA channel lifetimes (τ). (*C*) Changes in dimerization constant (left axis) and free energy of dimerization (right axis) for gA channel formation induced by 3 μM OA or DHA. τ_c, lifetime in the absence of fatty acid; τ, lifetime in the presence of fatty acid; f_c, channel appearance rate in the absence of fatty acid; f, appearance rate in the presence of fatty acid.

due to the difference in channel chirality because DHA has equal effect on the function of left- and right-handed channels of the same length [10 μM DHA increases the lifetimes of the enantiomeric AgA(15) and AgA$^-$(15) channels by 1.51- and 1.55-fold, respectively]. To a first approximation, gA channels' conformational preference does not vary as a function of bilayer thickness (41); the length dependence thus suggests that DHA alters gA channel function by a bilayer-mediated mechanism dependent on channel-bilayer hydrophobic mismatch (35, 42). To explore this further, we examined how the FA-induced changes in gA channel function varied as a function of bilayer thickness, channel length, and the presence of cholesterol, which increases the bilayer elastic moduli (43).

Fig. 3 shows changes in gA channel lifetime (τ) for different bilayer compositions at nominal [FA]s between 3 μM and 30 μM. At all [FA], DHA is a more effective modulator than OA. In cholesterol-free bilayers, the changes in channel function increase with increasing channel-bilayer hydrophobic mismatch, whether the channel length or the bilayer thickness is varied. In the presence of cholesterol, at a cholesterol:$DC_{18:1}PC$ molar ratio of 1:1, DHA is an even more effective modulator of channel function than in $DC_{18:1}PC$ bilayers, whereas OA remains inert. DHA also caused larger changes in the single-channel current than was the case in the absence of cholesterol. A 10 μM concentration of DHA caused a 15% increase in the absence and a 65% increase in the presence of cholesterol. Even OA increased the current: 10 μM OA caused a 5% increase in the absence and a 15% increase in the presence of cholesterol.

The different effects of DHA (and of OA) do not reflect different FA adsorption to the bilayers. The lipid bilayer/electrolyte partition coefficients (K_p) of both FAs were measured using ADIFAB (44). In cholesterol-free bilayers, K_p for OA was 10-fold greater than for DHA, with no variation as a function of acyl chain length (see Table 4). The greater "potency" of DHA, relative to OA, therefore is not due to greater adsorption to the bilayer; the bilayer thickness-dependent effects of DHA on the lifetime of AgA$^-$ (15) channels are due to changes in hydrophobic mismatch, not to changes in adsorption. Cholesterol had no effect on K_p for DHA, which is consistent with the results of Anel *et al.* (8) on other FAs, including OA.

Dimeric gA channels (D) form by transmembrane association of two nonconducting monomers (M):

$$2\mathrm{M} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{D}.$$

DHA increases both the channel appearance rate ($f = k_1 \cdot [\mathrm{M}]^2$) and lifetime ($\tau = 1/k_{-1}$), shifting the distribution between M and D (45):

$$\frac{[\mathrm{D}]}{[\mathrm{M}]^2} = \frac{k_1}{k_{-1}} = K_\mathrm{D} = \exp\left\{\frac{-\Delta G_{\mathrm{tot}}^{\mathrm{M}\to\mathrm{D}}}{RT}\right\}, \qquad [3]$$

where K_D is the dimerization constant, $\Delta G_{\mathrm{tot}}^{\mathrm{M}\to\mathrm{D}}$ is the total free energy of dimerization, R the gas constant and T temperature in kelvin. $\Delta G_{\mathrm{tot}}^{\mathrm{M}\to\mathrm{D}}$ can be expressed as the sum of two contributions: the intrinsic free energy change of dimerization (or protein "conformational change," $\Delta G_{\mathrm{def}}^{\mathrm{M}\to\mathrm{D}}$) and the free energy change of the bilayer deformation ($\Delta\Delta G_{\mathrm{def}}^{\mathrm{M}\to\mathrm{D}}$). Because the effect of DHA increases whether the bilayer thickness is increased or the channel length is decreased, we conclude that the changes in [D] result from changes in $\Delta\Delta G_{\mathrm{def}}^{\mathrm{M}\to\mathrm{D}}$ rather than from changes in

$\Delta G_{prot}^{M\to D}$. It thus becomes possible to estimate the DHA-dependent changes in $\Delta\Delta G_{def}^{M\to D}$ from the changes in f and τ because [D] = K_D·[M]^2 = f·τ (Fig. 4).

Fig. 4*A* shows changes in f induced by 3 μM DHA or OA for gA channels of varying lengths in bilayers of varying thicknesses (and in the absence and presence of cholesterol). (At 3 μM, bilayer breakage is relatively infrequent such that changes in channel appearance rate can be quantified in the same bilayer.) In parallel with the increase in f (Fig. 1), DHA (but not OA) also increases τ (Fig. 4*B*). Fig. 4*C* shows the relative increases in the time-averaged channel concentrations [D] (left ordinate), along with the corresponding changes in $\Delta\Delta G_{def}^{M\to D}$ (right ordinate). DHA increases K_D by a factor of 3–8, depending on the channel-bilayer hydrophobic mismatch, corresponding to decreases in $\Delta\Delta G_{def}^{M\to D}$ between 3 and 5 kJ/mol. In the stiffer $DC_{18:1}PC$:Chol (1:1) bilayers, $\Delta\Delta G_{def}^{M\to D}$ decreases by 6 kJ/mol, twice the change in $DC_{18:1}PC$ bilayers.

The changes in $\Delta\Delta G_{def}^{M\to D}$ could occur (cf. Eq. **1**) due to changes in bilayer thickness (d_0), bilayer elastic coefficients H_B, H_X, and H_C, lipid intrinsic curvature (c_0), or any combination thereof. We can exclude changes in d_0 because the bilayer specific capacitance does not vary by the addition of DHA or OA (being 4.1 ± 0.3 nF/mm^2 in the absence, and 4.3 ± 0.1 or 4.2 ± 0.2 nF/mm^2 in the presence of 10 μM DHA or OA, respectively). The effect of DHA on τ increases with increasing hydrophobic mismatch; we conclude that DHA decreases the disjoining force the bilayer imposes on the channels (cf. Eq. **2**) by reducing the magnitude of H_B (increasing bilayer elasticity).

DHA alters not only gA channel appearance rates and lifetimes, it also increases i (Fig. 2). These current changes could arise because adsorption of DHA at the bilayer/solution interface imparts a negative surface charge to increase [Na^+] at the channel entrance (and thus the single-channel current). DHA adsorption also may increase the "dielectric constant" due to the presence of the polyunsaturated acyl chains into the bilayer core, as the dielectric constant of hydrocarbons increase by ≈0.2 per double bond (46) (the single-channel current transition amplitudes are ≈10% higher in $DC_{18:2}PC$ as compared with $DC_{18:1}PC$ bilayers; ref. 47). Whatever the mechanism, the current changes provide insight into how DHA alters gA channel function.

Like other amphiphiles, DHA would be expected to alter global bilayer properties: to decrease the bilayer compression and bending moduli (30–32). In addition to these global effects, the gA-induced bilayer deformation may cause a local enrichment (or depletion) of DHA adjacent to the channel (ref. 48, cf. Fig. 5*A*), which would further reduce F_{dis} and $\Delta\Delta G_{def}^{M\to D}$. We can distinguish between these possibilities, enrichment vs. depletion, using the single-channel current changes as a measure of the local DHA surface density and examine how DHA increases i and τ in bilayers of different thickness. [As noted previously, the gA channel structure (41) does not vary with bilayer thickness.]

In the absence of DHA, changes in channel-bilayer hydrophobic mismatch do not affect the single-channel current transitions: in the case of AgA^-(15) channels, 3.40 ± 0.04 pA in $DC_{18:1}PC$ vs. 3.36 ± 0.04 pA in $DC_{20:1}PC$ bilayers (mean ± SEM, n = 6). The DHA-induced current changes, however, are larger in $DC_{20:1}PC$ as compared with $DC_{18:1}PC$ bilayers (Fig. 5*B*), indicating that the local [DHA] in the vicinity of the channels is higher in the thicker $DC_{20:1}PC$ bilayers; the relative lifetime changes vary as a linear function of the relative current changes, indicating that the changes in channel function depend, at least in part, on changes in local bilayer properties. Similar results were obtained with gA(13).

That the effect of DHA on both i and τ is larger in the thicker $DC_{20:1}PC$ bilayers provides additional support for the conclusion that the effect of DHA is not due to direct binding to the channels; if that were the case, the current (and relative lifetime) changes should not depend on bilayer thickness. We conclude that the DHA-induced changes in the free energy of gA channel formation arise because DHA modifies lipid bilayer material properties, in particular bilayer elasticity, and that the effect is enhanced by the local accumulation of DHA in the vicinity of the channel.

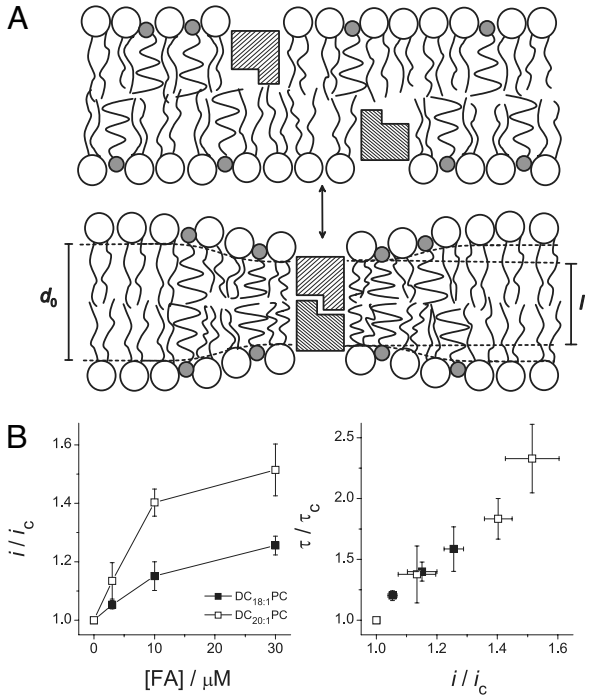


Fig. 5. DHA alteration of bilayer mechanical properties. (*A*) Schematic model in which DHA is enriched around the channel in the area of bilayer deformation. l is the channel hydrophobic length and d_0 the bilayer hydrophobic thickness. (*B*) Changes in AgA^-(15) channel properties by DHA in $DC_{18:1}PC$ (filled squares) and $DC_{20:1}PC$ (open squares). (*Left*) Normalized single-channel current (i) changes. (*Right*) Changes in τ as a function of changes in i.

Discussion

In this article, we extend the number of channels that are modulated by DHA and find, analogous to the situation for many other membrane proteins, that OA has little effect on gA channel function. The DHA-induced changes in gA channel function result from changes in bilayer elasticity, which stabilize the conducting dimer. Changes in material properties similarly may modulate the function of other membrane proteins; whether the modulation is activating or inhibiting depends on the $\Delta\Delta G_{def}^0$ for the particular conformational change. In the present context, we note that maneuvers that stabilize gA channels promote inactivation of voltage-dependent channels (35).

The DHA-induced changes in gA channel function occur at [FA] similar to those used in studies on integral membrane proteins (nominal concentrations of 1–10 μM, cf. the examples in Table 1). These concentrations are high compared with the unbound [FA] in plasma (49). The actual [FA] in the electrolyte solutions, however, are two orders of magnitude less [see supporting information (SI) *Text*]. At 3 μM DHA (or OA), for example, the FA mole fraction (m_{FA}) in the bilayer is ≈0.01 in the unperturbed bilayer, although m_{DHA} will be higher in the perturbed bilayer adjacent to the channel (cf. Fig. 5).

FAs promote the propensity to form inverse hexagonal phases (50) and cause negative-going changes in c_0 (34). Changes in c_0, *per se*, may cause changes in membrane protein function (34); ΔG_{def}^0, however, varies as a function of not only c_0 but also $d_0 - l$ and the elastic moduli, as expressed in the H coefficients (Eq.

BIOPHYSICS

1). If the changes in gA channel function predominantly were due to changes in c_0, then molecules that promote positive-going changes in c_0, such as Triton X-100 (35), should have effects opposite to those of DHA. That is not the case: both DHA and Triton X-100 (51) increase f and τ, and changes in c_0 cannot be the dominant mechanism underlying the changes in channel function.

The potency of DHA as a modifier of gA channel function increases with increasing channel-bilayer hydrophobic mismatch, whether one alters channel length or bilayer thickness (Figs. 3–5). The DHA-induced changes in channel function therefore do not result from direct channel-DHA interactions. The reduction in bilayer stiffness, as reported by the increases in f and τ, arises because DHA decreases the bilayer elastic moduli. Together with results on voltage-dependent sodium channels (35, 52), the present results provide further support for the notion that changes in bilayer elasticity constitute a general mechanism for modulating membrane protein function. Moreover, both PUFAs and lysophospholipids, which promote positive-going changes in c_0, are activators of the 2P domain potassium channels (53), providing additional support for the importance of changes in bilayer elasticity.

Finally, the results in Fig. 5 show that DHA is enriched in the vicinity of gA channels, whereas OA is not. Such local enrichment results because the free energy cost of the enrichment is balanced by a decreased bilayer deformation energy, and could occur because DHA with its six double bonds is more flexible than saturated or mono-unsaturated FAs (54, 55), which should enable DHA to pack more efficiently than OA into the perturbed bilayer region adjacent to a gA channel (membrane protein). This suggestion is consistent with the results of Feller *et al.* (56) who, based on molecular dynamics simulations on rhodopsin imbedded in 1-stearoyl-2-docosahexaenoyl-PC bilayers, found the docosahexaenoyl chain on average to be closer to rhodopsin than the stearoyl chain. Taken together, these findings highlight the difficulties associated with distinguishing "binding," due to short-range protein–ligand interactions, from "enrichment" due longer-range interactions, such as protein-induced bilayer perturbations. Indeed, even though PUFAs modulate gA channel function by a bilayer-mediated mechanism, the modulation is "specific" in the sense that some closely related molecules, such as OA, are inert. This "specificity," however, does not reflect chemically specific interactions, but rather the difference in physical properties of polyunsaturated vs. monounsaturated or saturated acyl chains. The distinctions among different FA effects therefore reflect the complex interplay between bilayer perturbations and local amphiphile accumulation in determining the energetic cost associated with protein conformational changes and the ensuing bilayer deformations.

Materials and Methods

Materials. gA analogues of different length and helix sense (Tables 2–4) were synthesized and purified as described (57). Stock solutions ($\approx 10^{-6}$ M) were stored in at −40°C and were diluted to 10^{-8} M to 10^{-9} M in absolute ethanol. The fatty acids (Table 4) were from Sigma (St. Louis, MO). For the electrophysiology experiments, stock solutions (0.5–5 mM plus an equimolar amount of NaOH) were made in absolute ethanol, stabilized with 25 μM butylated hydroxytoluene (BHT) and stored under argon. They were stable against oxidation for 2–3 weeks. For the determination of partition coefficients, stock solutions (0.5–5 mM) were made daily in 0.01 M NaOH without BHT and kept on ice. Acrylodated intestinal fatty acid binding protein (ADIFAB) was from Invitrogen (Carlsbad, CA) and was dissolved in 50 mM Tris, 1 mM EGTA, 0.05% azide, pH 8.0.

Table 2. gA sequences

Analogue	Sequence*	Hydrophobic Channel Length (nm)†
gA(13)	f-AL<u>A</u>V<u>V</u>V<u>W</u>L<u>W</u>L<u>W</u>LW-e	1.9
AgA(15)	f-A<u>G</u>AL<u>A</u>V<u>V</u>V<u>W</u>L<u>W</u>L<u>W</u>LW-e	2.2
AgA(17)	f-A<u>A</u>AGAL<u>A</u>V<u>V</u>V<u>W</u>L<u>W</u>L<u>W</u>LW-e	2.5

*The underlined residues are D-amino acids; f = formyl; e = ethanolamine.
†The hydrophobic length of the 15-aa gA channel is from ref. 58; the lengths of the other channels are adjusted by 0.3 nm per L-D pair of residues.

Table 3. Hydrophobic thickness of bilayers formed by different phospholipids

Phospholipid	Hydrophobic thickness, nm*
Dioleoylphosphatidylcholine ($DC_{18:1}PC$)	4.8
Dieicosenoylphosphatidylcholine ($DC_{20:1}PC$)	5.4

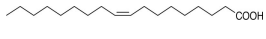
*Results from ref. 59.

Bilayer Formation and Single-Channel Measurements. Single-channel measurements were done as described (57) using 20 mg/ml solutions of dioleoylphosphatidylcholine ($DC_{18:1}PC$) or dieicosenoylphosphatidylcholine ($DC_{20:1}PC$) (Avanti Polar Lipids, Alabaster, AL) in *n*-decane (99.9% pure; Chemsampco, Trenton, NJ) across a ≈1.6 mm diameter hole in a 0.1-mm-thick Teflon partition separating two compartments filled with 1 M NaCl, 10 mM Hepes, pH 7.0 (Sigma). The current signal was recorded with an AxoPatch 1C patch clamp (Axon Instruments, Foster City, CA), filtered at 2,000 Hz, digitized and digitally filtered to between 100 and 500 Hz. Single-channel current transitions were detected as described (60).

The gA analogues were added to both sides of the bilayer. We used pairs of analogues having opposite helical sense to prevent heterodimerization. Except where noted, the FAs were added to both sides of the bilayer; the solutions were stirred for five min after addition and equilibrated for 10 min. (The final [BHT] was <0.1 μM; 0.5 μM BHT has no effect on channel function, results not shown.)

The current transitions for each channel type appear as a single peak in current transition amplitude histograms constructed as in ref. 60. For each channel type, lifetime histograms then were constructed for channels with current transitions falling within the characteristic peak in the amplitude histogram (see Fig. 2). The average channel lifetimes, τ, were determined by fitting a single exponential distribution $N(t) = N(0)\cdot e^{-t/\tau}$ [where $N(t)$ is the number of channels open longer than time t] to the lifetime distributions. The reported results are averages from at least three independent experiments, each with 300–1,000 channel events. Changes in channel appearance rate, f, were determined as the ratio of channel appearance rates from two 5- to 10-min recordings before and 10–20 min after OA or DHA addition (using only experiments where the bilayer did not break after FA addition).

Table 4. Partition coefficients of OA and DHA into different bilayers

Fatty acid	Structure	Partition coefficient ($\times 10^{-4}$) $DC_{18:1}PC$	+ Chol	$DC_{20:1}PC$
OA		254 ± 14		246 ± 28
DHA		20 ± 5	19 ± 2	20 ± 6

The membrane capacitance was measured using a sawtooth potential waveform (61).

Fatty Acid Partition Into Lipid Vesicles. Lipid bilayer/electrolyte FA partition coefficients (K_p) were determined in 1 M NaCl, 10 mM Hepes, pH 7.0 at 25 ± 1°C by ADIFAB (44) using a PerkinElmer (Salem, MA) 650–40 fluorescence spectrophotometer, exciting at 386 nm and measuring the fluorescence emission at 432 and 505 nm. After correcting for FA binding to the cuvette walls, the FA-ADIFAB dissociation constant (K_D) was determined.

The lipid-FFA partition coefficients were calculated as

$$K_p = \left(\frac{[FA]_1 - [FFA]}{[FFA]} \right) \frac{V_a}{V_m}, \qquad [4]$$

where $[FA]_t$ is total FA concentration, [FFA] is the free FA concentration, and V_m and V_a are the volumes of the membrane and aqueous solutions ($V_m/V_a \approx 10^{-3}$ per mM phospholipid; ref. 44). Lipid vesicles were prepared by miniextrusion (62) and diluted to ≈100 μM phospholipid and 0.2 μM ADIFAB was added; after each increase in $[FA]_t$ (3–30 μM), the system was equilibrated for 3–5 min and the fluorescence emission was measured. Each sample was assayed for the actual phospholipid concentration (63), and V_m/V_a was adjusted accordingly. For each [FA], K_p was determined in triplicate. K_p did not vary as a function of [FA]; the reported K_p values are averages over [FA] = 3–30 μM.

To estimate the FA adsorption of to the bilayer chamber, DHA or OA (3–30 μM) were added to a lipid-free Teflon chamber, stirred for 5 min and 0.5 ml was removed for [FFA] determination as above. The reported results are averaged over all measurements.

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