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Unique Effects of Different Fatty Acid Species on the Physical Properties of the *Torpedo* Acetylcholine Receptor Membrane*

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Silvia S. Antollini and Francisco J. Barrantes‡

From the Instituto de Investigaciones Bioquímicas de Bahía Blanca and UNESCO Chair of Biophysics and Molecular Neurobiology, B8000FWB Bahía Blanca, Argentina

To study the effects produced by free fatty acids (FFA) on the biophysical properties of *Torpedo marmorata* nicotinic acetylcholine receptor-rich native membranes and to investigate the topology of their binding site(s), fluorescence measurements were carried out using the fluorescent probe Laurdan (6-dodecanoyl-2-(dimethylamino) naphthalene) and ADIFAB, an Acrylodan-derivatized intestinal fatty acid-binding protein. The generalized polarization (GP) of the former probe was used to learn about the physical state of the membrane upon FFA binding. Saturated FFA induced a slight increase in GP, whereas *cis*-unsaturated fatty acids decreased GP. Double bond isomerism could also be distinguished; oleic acid (18:1*cis*) induced a net disordering effect, whereas elaidic acid (18:1*trans*) produced no changes in GP. The changes in the efficiency of the Förster energy transfer from the protein to Laurdan brought about by addition of FFA, together with the distances involved in this process, indicate that all FFA studied share a common site at the lipid-protein interface. However, despite being located at the same site, each class of FFA differs in its effect on the physical properties of the membrane. These data lead us to suggest that it is the direct action of FFA at the lipid-protein interface, displacing essential lipids from their sites rather than changes in bulk properties such as membrane fluidity that accounts for the effect of FFA on the acetylcholine receptor membrane.

The nicotinic acetylcholine receptor (AChR)[1] is an integral membrane protein deeply embedded in the postsynaptic region of muscle, electrocytes, and nerve cells. Experimental evidence from various groups including ours substantiates the notion that the function of this rapid ligand-gated channel is influenced by its lipid microenvironment (see reviews in Refs. 1–3). Although the occurrence of specific interactions between the transmembrane region of the AChR and adjacent lipid molecules in the membrane has been reported (4–6), the exact nature of these interactions has not been clearly established. The first shell of lipids around the AChR, the so-called annular lipid (7, 8), exhibits distinct characteristics, such as a higher degree of order and a lower mobility than the bulk lipids. This specialized region of the membrane has received particular attention as the likely candidate domain where modulation of AChR function by lipids occurs (7, 9).

The presence of both cholesterol and negatively charged phospholipids (10–16) has been shown to be necessary for proper AChR-mediated ion translocation *in vitro*. Various hypotheses were postulated to explain this functional dependence, because these lipids may modify the biophysical properties of the lipid annulus and/or the bulk lipid bilayer (12, 18, 19).

A second possibility is the occurrence of specific sites for certain lipids at the lipid-facing surface of the AChR, substantiated by the work of several groups (20–25). Upon interacting with these sites, lipids would stabilize the secondary structure of the AChR transmembrane segments (12, 26, 27). Recently, Baenziger *et al.* (28) proposed a model of how lipid composition modulates the function of the AChR, suggesting that membrane fluidity or some other bulk property of the membrane modulates the equilibrium between the resting and desensitized states of AChR. They also suggested that in addition to this indirect effect, the AChR has a specific requirement for anionic lipids (such as phosphatidic acid) binding to a specific site on the AChR or exerting a less specific charge effect on AChR conformation.

Previous studies from several laboratories demonstrate that free fatty acids (FFA) inhibit the ion flux mediated by the AChR *in vitro* (29, 30) or *in vivo* (31). Analysis of single-channel electrophysiological data argues for a mechanism compatible with noncompetitive inhibition of the AChR. From these studies the conclusion was drawn that the effect of FFA is related to their hydrophobic character, but the exact mechanism of FFA action is still not clear.

To further investigate the possibility that these compounds exert their effect on AChR at the lipid-protein interface and to investigate the nature of these effects on the biophysical property of the AChR-rich membrane, we carried out fluorescence studies using the fluorescent probes Laurdan (6-dodecanoyl-2-(dimethylamino)naphthalene) and ADIFAB, an Acrylodan-derivatized intestinal fatty acid-binding protein. Laurdan possesses an exquisite sensitivity to the phase state of the membrane. The physical origin of Laurdan spectral properties resides in its capacity to sense the polarity and the molecular dynamics of dipoles in its environment because of the effect of dipolar relaxation processes (32, 33). The principal dipoles sensed by Laurdan in the membrane are water molecules. When no relaxation occurs, high Laurdan GP values result, indicative of low water content in the hydrophobic/hydrophilic interface region of the membrane. Thus, GP values depend on the extent of water penetration allowed by the local membrane

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‡ To whom correspondence should be addressed. E-mail: rtfjb1@criba.edu.ar.

[1] The abbreviations used are: AChR, nicotinic acetylcholine receptor; *AHC*, actual hydrophobicity coefficient; FFA, free fatty acid(s); FRET, Förster resonance energy transfer; GP, generalized polarization.

packing and hence provide a direct report on the AChR membrane environment.

In the present work, we have exploited the advantageous spectroscopic properties of Laurdan to study the effect of FFA with different structure in the native membrane in which the AChR protein is embedded. Complementary studies with ADIFAB were also performed to determine the partition coefficient of the different fatty acids in the membrane. This information enabled us to compare the effects caused by fatty acids at the same effective concentration in the membrane. We found that the carbon chain length of fatty acids, as well as the number of double bonds and their stereochemical configuration, are important determinants of the unique effects of the different FFA species on the physical properties of the AChR-rich membrane. Furthermore, changes in the efficiency of the energy transfer from the protein to Laurdan, brought about by the addition of exogenous FFA, revealed the presence of sites for FFA at the lipid-protein interface in the native AChR membrane. Preliminary data have been presented in abstract form (34).

EXPERIMENTAL PROCEDURES

Materials

Torpedo marmorata specimens were obtained from the Mediterranean coast off Alicante, Spain. They were killed by pithing, and the electric organs were dissected and stored at −70 °C until further use. Laurdan and ADIFAB were purchased from Molecular Probes (Eugene, OR). All other drugs were obtained from Sigma.

Methods

Preparation of AChR-rich Membranes

Membrane fragments rich in AChR were prepared from the electric tissue of *T. marmorata* as described previously (35). Specific activities in the order of 2.0–2.8 nmol α-bungarotoxin sites/mg protein were obtained. The orientation of AChR in vesicles was measured as described by Hartig and Raftery (36) by determining the total toxin-binding sites in the presence of Triton X-100 and the right side out toxin-binding sites in the absence of detergent (36) as in previous work from our laboratory (37).

For fluorescent measurements, AChR-rich membranes were suspended in buffer A (150 mM NaCl, 0.25 mM $MgCl_2$, and 20 mM HEPES buffer, pH 7.4) at a final concentration of 50 μg protein/ml (0.2 μM). The optical density of the membrane suspension was kept below 0.1 to minimize light scattering.

Preparation of Fatty Acid Solutions

Sodium salts of FFA were dissolved in buffer A with a bath sonicator. In same cases we first prepared a sodium salt FFA stock solution in 4 mM NaOH, and aliquots were diluted to final concentrations with buffer A. FFA were dissolved in ethanol (in all cases the amount of ethanol added to the samples was kept below 0.5%).

Fluorescence Measurements

All fluorimetric measurements were performed in an SLM model 4800 fluorimeter (SLM Instruments, Urbana, IL) using a vertically polarized light beam from a Hannovia 200-W mercury/xenon arc obtained with a Glan-Thompson polarizer (4-nm excitation and emission slits) and 10 × 10-mm quartz cuvettes. Emission spectra were corrected for wavelength-dependent distortions. The temperature was set at 20 °C with a thermostated circulating water bath (Haake, Darmstadt, Germany).

Laurdan Measurements—Laurdan was added to AChR-rich membrane samples from an ethanol solution to give a final probe concentration of 0.6 μM. The amount of organic solvent was kept below 0.2%. The samples were incubated in the dark for 60 min at room temperature. Excitation GP (exGP) (32, 33) was calculated as follows.

$$\text{exGP} = (I_{434} - I_{490})/(I_{434} - I_{490}) \qquad \text{(Eq. 1)}$$

where I_{434} and I_{490} are the emission intensities at the characteristic wavelength of the gel phase (434 nm) and the liquid crystalline phase (490 nm), respectively. Excitation GP values were obtained from emission spectra obtained with an excitation wavelength of 360 nm.

Förster Resonance Energy Transfer (FRET) Measurements—The energy transfer efficiency (E) in relation to all other deactivation processes of the excited donor depends on the sixth power of the distance between donor and acceptor. According to Förster's theory (38), E is given by the following equation.

$$E = R_0^6/(R_0^6 + r^6) \qquad \text{(Eq. 2)}$$

where r is the intermolecular distance and R_o is a constant parameter for each donor-acceptor pair, defined as the distance at which E is 50%. E can be calculated as follows

$$E = 1 - (\phi/\phi_D) = 1 - (I/I_D) \qquad \text{(Eq. 3)}$$

where ϕ and ϕ_D are the fluorescence quantum yields of donor in the presence and absence of the acceptor, respectively, and I and I_D are the corresponding emission intensities in any given measurement. Here I and I_D correspond to the maximal intrinsic protein emission intensity, which is 330 nm.

When E was measured in the presence of exogenous FFA, a further correction was introduced to compensate for any modification of the intrinsic fluorescence of Trp by any other quenching mechanism induced by FFA.

$$E_{corr} = E_{(+\text{Laurdan})} - E_{(-\text{Laurdan})} \qquad \text{(Eq. 4)}$$

where E_{corr} is the experimentally determined value of E corrected by the quenching of the intrinsic fluorescence by the FFA. $E_{(+\text{Laurdan})}$ and $E_{(-\text{Laurdan})}$ values were calculated using Equation 3 in the presence of FFA, with or without Laurdan, respectively.

Partition Coefficient (K_p) Measurements—K_p was calculated with the following expression.

$$K_p = ([\text{FFA}]_m/[\text{FFA}]_a)\, V_a/V_m \qquad \text{(Eq. 5)}$$

where $[\text{FFA}]_m$ and $[\text{FFA}]_a$ are the concentrations of fatty acid in the membrane phase and in the aqueous phase, respectively, and V_a and V_m are the corresponding volumes of the aqueous and membrane phases, respectively. $[\text{FFA}]_m$ is defined as $[\text{FFA}]_T - [\text{FFA}]_a$, where $[\text{FFA}]_T$ is the added FFA concentration. Experimentally, the K_p values were obtained using ADIFAB, which responds to FFA binding with a shift in fluorescence emission from 432 nm in the apoform to 488 nm in the holoform. As a consequence, $[\text{FFA}]_a$ can be determined from the ratio of the fluorescence intensity at 488 nm to that at 432 nm (39), according to the following expression.

$$[\text{FFA}]_a = K_d Q(R - R_0)/(R_{max} - R) \qquad \text{(Eq. 6)}$$

where R is the measured ratio of 488 to 432 nm intensities, R_o is this ratio with no FFA present, R_{max} is the value when ADIFAB is saturated with FFA, and $Q = I_F(432)/I_b(432)$, where $I_F(432)$ and $I_b(432)$ are the ADIFAB intensities with zero and saturating concentrations of FFA, respectively. On the basis of the numerical analysis of FFA titration data, Q and R_{max} values were found to be 19.5 and 11.5, respectively (40). These values are constant for all FFA. K_d is the dissociation constant for FFA (ADIFAB-FFA ⇔ ADIFAB + FFA), calculated from a plot obtained by titration of ADIFAB with FFA, after linearization according to a logarithmic form of Equation 6 (Hill plot). K_d values were obtained using the following expression.

$$[\text{FFA}]_a = [\text{FFA}]_T - [\text{ADIFAB}] \times \{A/(1 + A)\} \qquad \text{(Eq. 7)}$$

where $A = Q(R - R_o)/(R_{max} - R)$ and [ADIFAB] was 0.2 μM.

Determination of the Effective FFA Concentration in Native Torpedo AChR-rich Membrane—The $[\text{FFA}]_T$ for each addition was converted to its effective concentration inside the membrane ($[\text{FFA}]_e$) using the following equation.

$$[\text{FFA}]_e = C_L \times [\text{FFA}]_T \qquad \text{(Eq. 8)}$$

where $C_L = K_p/\{1 + (K_p - 1)\, \gamma_L\, [L]\}$, γ_L is the lipid molar volume, and [L] is the lipid concentration in the cuvette, which increases for each addition of FFA. A value of 0.95 dm^3/L (dipalmitoylphosphatidylcholine fluid phase) was assumed because AChR-rich membranes are in a liquid fluid phase at 20 °C (41).

Data Analysis

Intergroup comparisons were done by impaired t test. Statistical significance was accepted as $p < 0.05$.

RESULTS

Partition of Free Fatty Acids in AChR-rich Membranes—To compare the effects caused by the presence of different exoge-

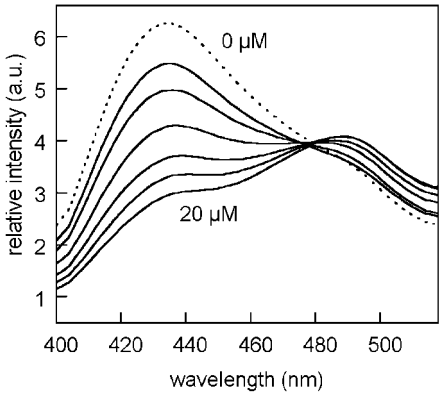

FIG. 1. **Changes in ADIFAB fluorescence emission spectrum by titration of AChR-rich membrane from *T. marmorata* with arachidonic acid.** The *dotted line* corresponds to the spectrum of ADIFAB in its apoform (maximum at 432 nm); the *solid lines* are the spectra of ADIFAB in the presence of increasing amounts (up to 20 μM, from *top* to *bottom*) of arachidonic acid. A diminution of the emission maximum at 432 nm and an increase of a second emission maximum at 488 nm is apparent.

TABLE I
Fatty acid-ADIFAB dissociation constants (K_d) and fatty acid-native Torpedo AChR-rich membrane partition coefficients (K_p)

Fatty acid	K_d	K_p ($\times 10^{-4}$)
Stearic (18:0)	0.49	114.5 ± 17
Arachidic (20:0)	0.72	156.5 ± 12
Oleic (*cis* 18:1)	0.26	54.6 ± 10
Elaidic (*trans* 18:1)	0.26	66.9 ± 20
Linoleic (18:2)	2.40	7.0 ± 0.5
Linolenic (18:3)	4.32	2.7 ± 0.1
Arachidonic (20:4)	2.21	8.2 ± 1.0
Docosahexanoic (22:6)	1.96	10.8 ± 0.7

nous FFA on the native AChR-rich membrane from *T. marmorata*, we first measured their partition coefficient. There are several methods for measuring partition coefficients of lipids in membranes, but the clear advantage of using ADIFAB is that it is not necessary to physically separate free and membrane-bound fatty acid, as is the case when using radiolabeled fatty acids, a method more prone to error. ADIFAB, an Acrylodan-tagged intestinal fatty acid-binding protein (39, 40), is a suitable fluorescent indicator for the measurement of FFA concentration in the 1 nM to 20 μM range. The detection of FFA by ADIFAB is based on a change in the position of the Acrylodan fluorescent tag relative to the nonpolar binding pocket of the protein when the latter is occupied by a fatty acid. ADIFAB undergoes a marked spectral shift upon FFA binding, allowing the determination of FFA concentrations from the ratio of the fluorescence intensities of bound and unbound forms, measured at about 488 and 432 nm, respectively. In the example shown in Fig. 1, the decrease in intensity at 432 nm and the corresponding increase at 488 nm of the ADIFAB spectrum is apparent upon titration of AChR-rich membrane from *T. marmorata* with arachidonic acid.

Using Equations 6 and 5 we obtained the values of the dissociation constant (K_d) and partition coefficient (K_p) for the different fatty acids and ADIFAB in aqueous solution and in the presence of *Torpedo* AChR-rich membranes, respectively (Table I). The calculated K_p values allowed us to classify the fatty acids into three different groups: (i) highly hydrophobic fatty acids, such as 20:0 and 18:0; (ii) less hydrophobic fatty acids, such as 18:1*cis* and 18:1*trans*, and (iii) more hydrophilic fatty acids, such as 18:2, 18:3, 20:4, and 22:6.

The experimentally determined K_p values of the fatty acids in native AChR-rich membrane were subsequently used to transform the nominal concentration added in the cuvette into an effective concentration in the membrane for each fatty acid (see "Experimental Procedures").

Variations of Laurdan GP Values Caused by the Addition of Free Fatty Acids to AChR-rich Membranes of T. marmorata—To study the modification of the physical properties of the *Torpedo* native membrane induced by the presence of FFA, we exploited the amphiphilic fluorescence probe Laurdan's exquisite sensitivity to the phase state of the membrane. We have previously used the so-called GP of Laurdan as a sensitive tool to measure the physical state of *Torpedo* native AChR-rich membrane (25, 41).

In the present work we investigated the possible modification of the polarity of both the AChR belt and bulk lipid regions by fatty acids. Laurdan GP values were obtained by direct excitation (at 360 nm) or under FRET conditions, using the intrinsic membrane fluorescence as donor (excitation at 290 nm) and Laurdan molecules as acceptor. In a previous work we characterized this system and showed that the GP values obtained under FRET conditions exhibit higher absolute GP values than those obtained by direct excitation of the probe, indicating that the microenvironment of the AChR has lower polarity than the bulk lipid (41).

The changes in GP values caused by different FFA could be compared using the effective concentration of each fatty acid inside the membrane, calculated using their K_p values (Fig. 2). We found that addition of FFA to *Torpedo* native membrane changed GP values, albeit to different extents, in a manner dependent on the chemical structure of the fatty acids.

Unsaturated fatty acids decreased GP values, whereas saturated ones caused a small increase. Statistically significant differences were observed between different fatty acids: 18:0–18:1*cis* ($p < 0.005$); 18:1–18:2 ($p < 0.001$); 18:2–18:3 ($p < 0.001$); 18:2–20:4 ($p < 0.005$); 18:2–22:6 ($p < 0.001$); 18:3–20:4 ($p < 0.001$); 18:3–22:6 ($p < 0.001$); and 20:4–22:6 ($p < 0.025$), the exception being 18:0–20:0 ($p > 0.05$). In a fatty acid, each double bond induces a kink of nearly 30° in the acyl chain (42). Thus, a fatty acid with several double bonds undergoes noticeable changes in the direction of its main molecular axis, resulting in an increase in the cross-sectional area of the hydrocarbon chain over the minimum found in a saturated fatty acid. The decrease in the GP values induced by unsaturated FFA indicates an increase in polarity within the Laurdan microenvironment. This in turn is a clear reflection of the disordering of the bilayer caused by the kinked chains of unsaturated fatty acids, which increases the content of water molecules in the membrane. In contrast to unsaturated FFA, saturated FFA induce only a small increase in GP and thus a slight decrease in polarity and hence in the amount of water in the membrane. In other words, saturated fatty acids induce a slight ordering effect in the membrane, probably because of their rigid linear structure.

The effect of fatty acid isomerization on GP was studied next. A notable difference was observed in the effect caused on Laurdan GP by 18:1*cis* (oleic acid) and 18:1*trans* (elaidic acid) (Fig. 3). Whereas oleic acid decreased GP values, elaidic acid caused no changes. Again, the difference in molecular structure of these fatty acids is a plausible explanation for this behavior, because as stated above, *cis*-double bonds induce a kink in the chain, whereas *trans*-double bonds produce no change in chain direction (Fig. 3, *inset*).

Similar Sites for Fatty Acids at the AChR-Lipid Interface—In a previous work we were able to discriminate between distinct sites for phospholipids and sterols, both accessible to fatty acids, at the lipid-protein interface in *Torpedo* native membrane, by measuring the decrease of E between the membrane intrinsic fluorescence (tryptophan residues) and Laurdan upon

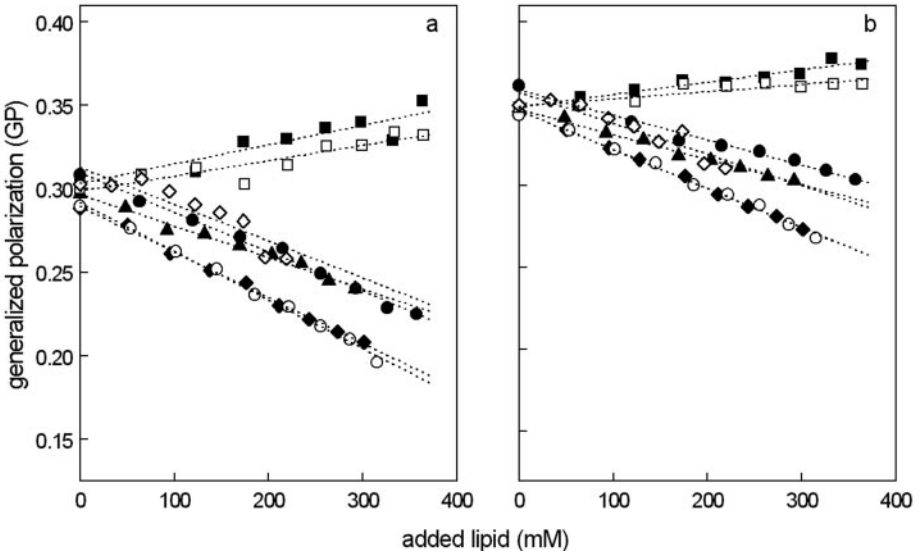

FIG. 2. **Laurdan excitation GP using direct excitation of the probe (360 nm) (*a*) or FRET conditions (290 nm) from the protein emission in *T. marmorata* AChR-rich membrane (*b*) in the presence of increasing concentrations of stearic acid (■), arachidic acid (□), oleic acid (●), linoleic acid (▲), linolenic acid (◇), arachidonic acid (◆), and docosahexaenoic acid (○).** The *abscissa* shows effective fatty acid concentration in the membrane, calculated using the K_p of each FFA and Equation 8 (see "Experimental Procedures"). Each point is the average of at least four independent measurements (see text for statistics).

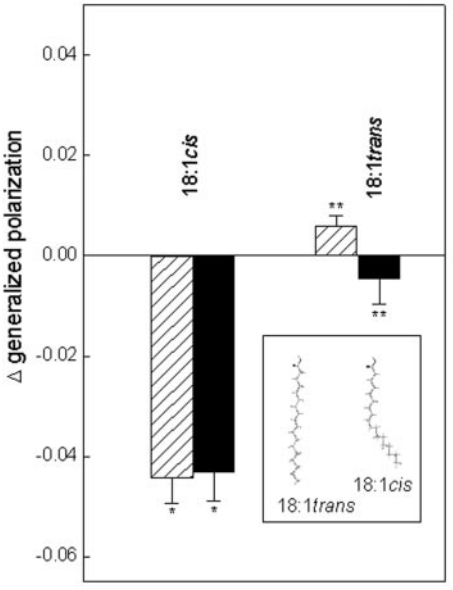

FIG. 3. **Net variation in Laurdan GP (Δ*GP*) in *T. marmorata* AChR-rich membrane obtained between GP values in the presence of 20 μM oleic acid (18:1*cis*) or elaidic acid (18:1*trans*) on the one hand and GP values with addition of only buffer on the other.** *Striped* and *black bars* correspond to the direct excitation or FRET conditions, respectively. Each point is the average of at least four independent measurements. *, statistically significant differences with respect to control values and with respect to 18:1*trans* (in both cases $p < 0.001$); **, nonsignificant differences. *Inset*, molecular structure of oleic and elaidic acids.

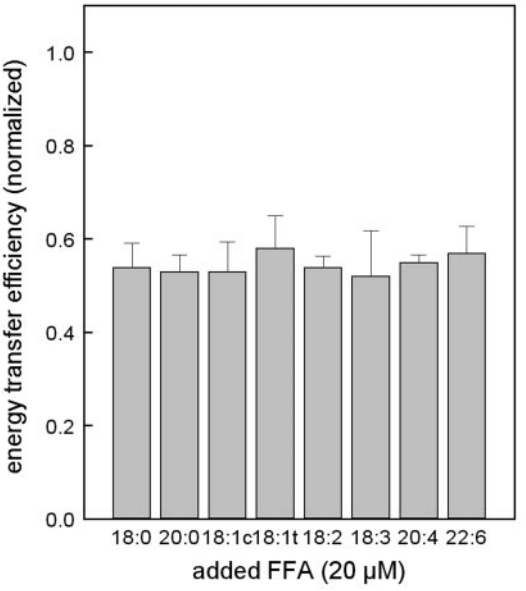

FIG. 4. **Decrease in normalized FRET efficiency (*E*) between intrinsic fluorescence in native *T. marmorata* AChR-rich membrane and Laurdan in the presence of maximal concentrations (20 μM) of stearic acid (18:0), arachidic acid (20:0), oleic acid (18:1*cis*), elaidic acid (18:1*trans*), linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4), and docosahexaenoic acid (22:6).** The *E* values obtained in the presence of fatty acid were normalized with respect to the corresponding *E* value in its absence (100%). Each point corresponds to the average ± S.D. of four determinations.

addition of different exogenous lipids (25). Here we measured changes of *E* between AChR and Laurdan, brought about by the addition of FFA of different chain length and degree of saturation. The addition of FFA decreased the efficiency of FRET in a concentration-dependent manner. The maximal diminution of *E* achieved by all fatty acids was very similar (~ 50%) (Fig. 4). This suggests that all fatty acids, independently of their physical characteristics, bind to similar sites at the lipid-protein interface in the *Torpedo* AChR-rich native membrane.

To corroborate whether different FFA bind to similar sites, we conducted an additional series of experiments using the following strategy. We added first a given FFA, and, after obtaining the maximal decrease of *E*, we added a second FFA, with similar or different structural characteristics, to the sample. If different FFA bind to different sites, *E* should further decrease when a second FFA is added, whereas no additional decrease of *E* would occur if the two FFA compete for the same site. Fig. 5 (*a* and *b*) shows one such series of experiments. When, for example, 20:0 was added first, Laurdan GP increased. When a second FFA was added, changes in GP depended on its chemical structure; 18:0 caused a further *increase* in GP, whereas 18:1 and 20:4 *decreased* GP values to the same extent as they did by themselves separately. The differences between GP values obtained after addition of different fatty acids were in all cases statistically significant. Only in the case of the saturated fatty acids 18:0 and 20:0 did we find statistically nonsignificant differences. The fact that Laurdan GP was affected under FRET conditions (Fig. 5*b*) is a clear indication that the second FFA partitioned well into the membrane and localized in the AChR-vicinal region. The decrease of

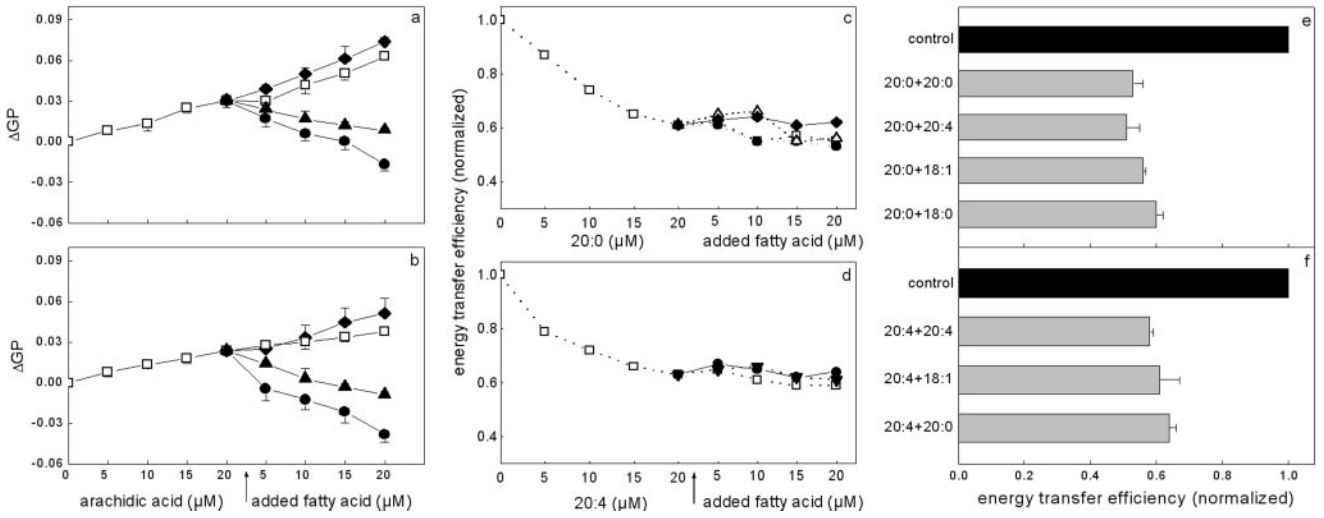

FIG. 5. **Net variation of Laurdan GP using direct excitation of the probe (*a*) and FRET conditions (*b*) first in the presence of increasing concentrations of arachidic acid (20:0, □) and then in the presence of stearic acid (18:0, ♦), oleic acid (18:1, ▲), or arachidonic acid (20:4, ●).** *c* and *d*, normalized energy transfer efficiency for the AChR/Laurdan pair in *T. marmorata* membranes. Two different conditions are shown here: increasing concentrations of 20:0 up to 20 μM (*c*) and increasing concentrations of 20:4 up to 20 μM (*d*). Subsequently, a second fatty acid was added up to a concentration of 20 μM. *c*, 20:0 (□), 20:4 (●), 18:1 (△), and 18:0 (♦) were added. *d*, 20:4 (□), 18:1 (▼), and 20:0 (●) were added. *e* and *f*, final values of FRET *E* attained upon addition of a second fatty acid. The differences between experimental conditions were statistically not significant. These results correspond to the averages ± S.D. of at least four independent measurements.

E obtained with the first fatty acid (saturated or unsaturated) remained constant in the presence of a second fatty acid, independently of its physical characteristics (Fig. 5, *c–f*).

DISCUSSION

In the present work, we used fluorescence techniques to study the modifications of the physical properties of native *Torpedo* AChR-rich membrane by the addition of fatty acids with different structural characteristics. We first determined the partition coefficient of different FFA using an Acrylodan-labeled fatty acid-binding protein, ADIFAB. This enabled us to calculate the effective concentration of the FFA in the membrane. Quantification of Laurdan GP yielded information on the physical state of the bulk and belt-lipid region of the AChR-rich membrane in the presence of known concentrations of FFA. Changes in FRET efficiency induced by fatty acids made possible the measurement of fatty acid-protein interactions within short distances of the probe Laurdan.

The FFA partition coefficients, determined using the extrinsic fluorescent properties of ADIFAB, made apparent relatively pronounced differences in K_p between different fatty acids (Table I). Although FFA are highly hydrophobic compounds, their physicochemical properties have a common origin: their carbon atoms confer hydrophobicity, and the double bonds reduce hydrophilicity in the molecule (42). We have attempted to relate these two opposite effects in the same molecule by using an empirical algorithm that we have coined the actual hydrophobicity coefficient (*AHC*).

$$AHC = \frac{\text{number of C/FFA} - \text{number of C in double bond/FFA}}{\text{number of C of the longest FFA}} \quad \text{(Eq. 9)}$$

We calculated this coefficient for all FFA studied and correlated it with their partition coefficient (Fig. 6). Using this approach, it is observed that the tendency of different FFA to partition in the membrane follows the sequence: 20:0 > 18:0 > 18:1 > 18:2 ≅ 20:4 ≅ 22:6 > 18:3. According to this order, FFA can be classified into three different groups: (i) high *AHC* fatty acids, such as 20:0 and 18:0; (ii) fatty acids of intermediate *AHC*, such as 18:1*cis* and 18:1*trans*; and (iii) low *AHC* fatty acids, such as 18:2, 18:3, 20:4, and 22:6.

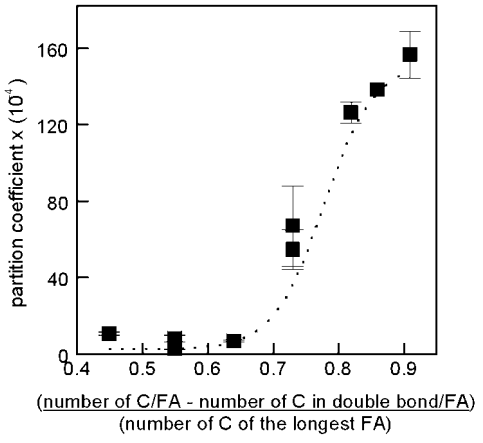

FIG. 6. **Correlation between hydrophobicity/hydrophilicity of fatty acids and their K_p in *Torpedo* native membrane.**

All fatty acids tested decreased the efficiency of the energy transfer, *E*, between the membrane intrinsic fluorescence and Laurdan. In a previous work (25), we interpreted this diminution as an increase in the distance between donor and acceptor resulting from the displacement of Laurdan molecules from the AChR lipid microenvironment caused by the exogenously added lipid (Fig. 7). We were also able to characterize distinct sites for phospholipids, sterols, and oleic acid. Here, we investigated whether the sites to which oleic acid binds are the same as those to which other fatty acids bind. The fact that a similar decrease in *E* was observed for all FFA is a strong indication that this is indeed the case. Further evidence in support of the above hypothesis is that the addition of a second fatty acid did not produce a further decrease of the *E* caused by the first fatty acid.

The most striking finding of the present work is that although all fatty acids displaced a fatty acid analog such as Laurdan to almost the same extent (Fig. 4), different fatty acids perturbed the physical properties of native *Torpedo* AChR-rich membranes quite differently, in a manner that strongly depended on their structure (Fig. 2). Thus, saturated fatty acids

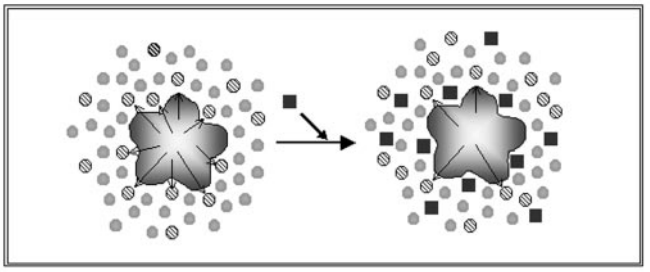

FIG. 7. **Schematic diagram illustrating a cross-section of the membrane containing Laurdan molecules in the belt and bulk lipid regions, showing the displacement of Laurdan molecules ([hatched circle]) from the AChR microenvironment by exogenously added fatty acids (■).**

ordered the membrane, whereas unsaturated fatty acids disordered it. Applying a simple algebraic rearrangement of the changes in GP made it possible to classify FFA effects.

$$\text{FFA effect} = (GP_{\text{final}} - GP_{\text{initial}}/(GP_{\text{g}} - GP_{\text{1c}}) \qquad \text{(Eq. 10)}$$

where GP_{final} and GP_{initial} are the GP values obtained at the same fatty acid effective concentration in the membrane and before addition of the fatty acid, respectively, and GP_{g} and GP_{lc} are the larger and smaller GP values obtained for a pure gel and liquid crystalline phase, respectively. GP_{g} (0.61) and GP_{lc} (−0.19) were experimentally measured at 20 °C in multilamellar DOPC and dipalmitoylphosphatidylcholine liposomes, which are in the liquid crystalline and gel phases, respectively.

Applying Equation 10, the changes induced by FFA ranged from ordering (positive values of FFA effect) to disordering (negative values of FFA effect) effects and followed the sequence: 20:0 (0.016) > 18:0 (0.005) > 18:1*cis* (−0.047) ≅ 18:2 (−0.046) ≅ 20:4 (−0.047) ≅ 22:6 (−0.047) > 18:3 (−0.056). Furthermore, whereas 18:1*cis* (oleic acid) induced a net disordering effect, 18:1*trans* (elaidic acid) produced no change whatsoever in GP values (Fig. 3). This enables us to conclude that FFA carbon chain length as well as the number of double bonds and their stereochemical configuration are important determinants of the unique effects of the different FFA on the physical properties of the *Torpedo* AChR-rich membrane.

In living BC3H-1 cells in culture, fatty acids exert inhibitory effects on AChR channel activity (31). These effects are also observed in membrane patches excised from the cell and are therefore not mediated by signal transduction pathways that require soluble factors such as nucleotides and Ca^{2+}. Thus, fatty acids appear to regulate the action of the AChR channel directly, much as they regulate the action of several purified enzymes and other channels (43). In chick ciliary neurons and in α7 neuronal type AChR heterologously expressed in *Xenopus* oocytes, saturated FFA or with only one double bond had little effect on ACh-mediated currents, whereas FFA with two or three double bonds produced partial inhibitory effects but less effectively than arachidonic acid (44). The latter FFA has also been reported to inhibit ACh-mediated currents in bullfrog sympathetic neurons (17). The mechanism by which 20:4 or other fatty acids inhibit nicotinic transmission is unknown. To account for these inhibitory effects on AChR function, binding of fatty acid to a site(s) at the lipid-AChR interface and/or perturbation of the local receptor microenvironment have been suggested.

Structurally different fatty acids (i) all appear to alter AChR function in a similar manner, in terms of single channel open channel durations at the level of resolution achieved (31); (ii) occupy equivalent sites at the lipid-protein interface, in fact the same sites as cholesterol and phospholipids (25); and (iii) induce changes in the fluidity of the AChR lipid microenvironment that clearly depend on their chemical structure. Thus, an explanation of fatty acid action mediated only through fluidity changes is difficult to sustain, because different fatty acids induce changes in membrane fluidity of different sign and magnitude; some in fact induce no change at all. We suggest that it is the direct action of FFA in displacing essential lipids from their sites at the lipid-protein interface and not changes in bulk properties such as membrane fluidity that is responsible for the inhibitory effect of FFA on AChR function.

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