Modulation of Nicotinic Acetylcholine Receptor Conformational State by Free Fatty Acids and Steroids*

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Steroids and free fatty acids (FFA) are noncompetitive antagonists of the nicotinic acetylcholine receptor (AChR). Their site of action is purportedly located at the lipid-AChR interface, but their exact mechanism of action is still unknown. Here we studied the effect of structurally different FFA and steroids on the conformational equilibrium of the AChR in Torpedo californica receptor-rich membranes. We took advantage of the higher affinity of the fluorescent AChR open channel blocker, crystal violet, for the desensitized state than for the resting state. Increasing concentrations of steroids and FFA decreased the $K_D$ of crystal violet in the absence of agonist; however, only cis-unsaturated FFA caused an increase in $K_D$ in the presence of agonist. This latter effect was also observed with treatments that caused the opposite effects on membrane polarity, such as phospholipase A$_2$ treatment or temperature increase (decreasing or increasing membrane polarity, respectively). Quenching by spin-labeled fatty acids of pyrene-labeled AChR reconstituted into model membranes, with the label located at the M4 transmembrane segment, disclosed the occurrence of conformational changes induced by steroids and cis-unsaturated FFA. The present work is a step forward in understanding the mechanism of action of this type of molecules, suggesting that the direct contact between exogenous lipids and the AChR transmembrane segment removes the AChR from its resting state and that membrane polarity modulates the AChR activation equilibrium by an independent mechanism.

Ligand-gated ion channels belong to a superfamily of proteins of which the muscle nicotinic acetylcholine receptor (AChR)$^2$ is the best-characterized member. It is an integral membrane protein deeply embedded in the postsynaptic region, with two agonist binding sites and a central ion pore (1). A variety of high affinity noncompetitive antagonists (NCAs) of the AChR exert their action mainly through the ion pore. Some bind to the resting state of the AChR (tetracaine and 3-trifluoro-4-methyl-3-(m-iodophenyl)diazirine), whereas others (e.g. phencyclidine, ethidium, chlorpromazine) preferentially bind to the desensitized form at equilibrium. Compounds such as these are predominantly hydrophilic molecules, their site of action being located in the extracellular part of the protein (for review, see Ref. 2). A group of highly hydrophobic compounds (free fatty acids (FFA) and steroids) behave as low affinity NCAs of the AChR. Several functional studies on muscle and neuronal AChR demonstrate this inhibitory behavior (for FFA, see Refs. 3–9; for steroids see Refs. 10–25).

Recently, we demonstrated that both exogenous and endogenous FFA and steroids localize inside the membrane, binding to common sites at the lipid-AChR interface (26). These molecules are likely to modulate the AChR function by a mechanism of action different from that exerted by hydrophilic inhibitors. For ligands acting at the lipid-protein interface, essentially two mechanisms of AChR inhibition can be considered, (a) displacement of an essential lipid from the protein-lipid interface or (b) changes in the physical properties of the lipid bilayer.

The muscle AChR is a pentamer composed of four different but homologous subunits in the stoichiometry $\alpha_2\beta_2\gamma\delta$. Each subunit contains a relatively large extracellular domain and four hydrophobic segments referred to as M1–M4, proposed to be membrane-spanning segments, and ends with a short extracellular carboxyl-terminal domain. Three concentric rings can be distinguished in the AChR transmembrane (TM) region (27, 28). The M2 TM segments of all subunits outline the inner ring and form the walls of the ion channel proper; M1 and M3 constitute the middle ring, and the M4 segments form the outer ring, which is in closest contact with the AChR lipid environment. Thus, an important portion of the AChR surface is exposed to the TM region, in contact with the surrounding lipids, establishing cross-talk between lipids and the protein moiety (27, 28). Subunit interactions, which are crucial for AChR activation, have been postulated to involve not only the AChR extracellular domain but also TM domains (29).

Marsh and Barrantes (30) identified a layer of immobilized lipids surrounding the AChR distinct from the bulk lipids and postulated this layer as a possible site of pharmacological action. Criado et al. (31) followed the kinetics of agonist-induced state transitions and showed that AChR function is sensitive to its lipid environment, postulating that the full function requires the presence of cholesterol or analogs. Sunshine and

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3 The abbreviations used are: AChR, nicotinic acetylcholine receptor; Carb, carbamoylcholine; CrV, crystal violet; FFA, free fatty acids; sFFA, saturated FFA; cis-FFA, cis-unsaturated FFA; HC, hydrocortisone; NCA, non-competitive antagonist; N-PyrM, N-(1-pyrenyl)maleimide; PLA$_2$, phospholipase A$_2$; 5-SLFA, 5-spin labeled fatty acid; TM, transmembrane; FRET, Förster-type resonance energy transfer; GP, generalized polarization.
McNamee (32), reconstituting the AChR in different lipid environments, concluded that the lipid composition is more important than the bulk membrane fluidity in determining the AChR ion channel function; however, they subsequently indicated that the specific molecular structure of cholesterol was not key for sustaining AChR function and pointed to the hydrophobicity of the neutral lipids as the major factor in supporting AChR activity (33). They also found a marked lipid dependence on the rate of agonist-induced desensitization, suggesting that the membrane lipid environment can directly affect the conformational changes responsible for desensitization. Different combinations of lipids appear to induce the same, final desensitized conformation of the AChR as produced by prolonged exposure to agonist (34). Narayanaswami et al. (35) postulated that relatively fluid layers of lipid surrounding the AChR, probably with an optimal fluidity, are responsible for facilitating AChR conformational changes. Studies from our laboratory indicated the presence of distinct sites at the AChR lipid interface for cholesterol and phospholipids (36).

In this paper we studied the effect of structurally different FFA and steroids on the AChR conformational equilibrium in native Torpedo californica membranes using a fluorescent dye that has a higher affinity for the desensitized state of the AChR in an attempt to understand the effect of these two types of hydrophobic molecules on the modulation of the AChR and to discriminate between direct and indirect modes of action. The results indicate that there are two levels of AChR conformational modulation. First, the mere presence of FFA or steroids can directly drive the AChR out of its native-resting-conformation; second, perturbations of membrane polarity prevent the AChR from reaching the agonist-induced desensitized state.

**EXPERIMENTAL PROCEDURES**

**Materials**

*T. californica* specimens obtained from the Pacific coast of California were killed by pithing, and the electric organs were dissected and stored at −70 °C until further use. Laurdan and pyrene-maleimides were purchased from Molecular Probes (Eugene, OR). Phospholipase A$_2$ (PLA$_2$) was obtained from Roche Applied Science. Affi-Gel® 10 Gel and dithiothreitol were obtained from Bio-Rad. Synthetic lipids and 5-spin labeled fatty acid (5-SLFA) were from Avanti Polar Lipids, Inc. (Birmingham, AL). Crystal Violet (CrV) and all the others drugs were obtained from Sigma-Aldrich.

**Methods**

**Preparation of AChR-rich Membranes**—Membrane fragments rich in AChR were prepared from the electric tissue of *T. californica* as described previously (37). Briefly, electric tissue was chopped into small pieces, homogenized under controlled conditions using a Virtis homogenizer, and submitted to a series of centrifugation steps ending in a high speed sucrose gradient centrifugation. Specific activities on the order of 2.0–2.8 nmol of α-bungarotoxin sites/mg of protein were obtained in the middle, AChR-rich membrane fraction (37). The orientation of AChR in the membrane vesicles was determined as described by Hartig and Raftery (38) by comparing 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 as in previous work from our laboratory (39).

For the fluorescence measurements, AChR-rich membranes and N-PyrM-AChR 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 100 μg of protein/ml (0.4 μM) or 20 μg/ml, respectively. The optical density of the membrane suspension was kept below 0.1 to minimize light scattering.

**Preparation of Steroid and Free Fatty Acid Solutions**—Cortisone and hydrocortisone (HC) were dissolved in (1:1) ethanol: DMSO. FFAs were dissolved in ethanol. In all cases the amount of organic solvent added to the samples was kept below 0.5%. After each steroid or FFA addition, samples were kept at 25 °C for 30 min to allow equilibration of the added hydrophobic compounds with the membrane.

**Fluorescence Measurements**—All fluorimetric measurements were performed in an SLM model 4800 fluorimeter (SLM Instruments, Urbana, IL) using a vertically polarized light beam from Hannovia 200-W mercury/xenon arc obtained with a Glan-Thompson polarizer (4-nm excitation and emission slits) and 1-ml quartz cuvettes. The temperature was set at 25 °C with a thermostatted circulating water bath (Haake, Darmstadt, Germany) except when indicated otherwise.

**CrV Fluorescent Measurements**—CrV was dissolved in buffer A at three different stock concentrations (10, 50, and 350 μM) and stored at −20 °C for posterior titration experiments. For the fluorescence measurements, AChR-rich membranes were suspended in buffer A. The samples were titrated with CrV. After each addition, the samples were incubated for 15 min to allow equilibration, and then the emission spectra were collected. CrV was excited at 600 nm, and the fluorescence emission spectra were collected from 605 to 700 nm. A background spectrum (obtained from the same cuvette before CrV addition) was subtracted from the emission spectra obtained in the presence of CrV, and the maximum intensity (at 623–625 nm) was measured. To determine the CrV dissociation constants (K$_D$), the value of CrV maximum fluorescence emission was plotted as a function of the logarithmic CrV concentration (molarity), the resulting sigmoid curve was fitted to the Boltzmann function, and the K$_D$ was calculated.

To test the magnitude of the nonspecific drug binding (40), we also undertook control experiments using membrane fractions from *T. californica* devoid of AChR. After obtaining the crude membrane fraction by differential centrifugation, an additional sucrose gradient centrifugation was performed, and three distinct membrane bands were obtained (37). The middle band is highly enriched in AChR (“AChR-rich membranes”), whereas the top band has less AChR protein, and the bottom band is devoid of AChR protein. Thus, the bottom band was used as a control for CrV unspecific labeling (see the supplemental figure). The nonspecific signal was found to be small and, therefore, does not introduce significant changes in the results showing the K$_D$ of CrV.

**PLA$_2$ Treatment of AChR-rich Membranes**—AChR-rich membranes were suspended at a final concentration of 1 mg/ml in phosphate buffer (10 mM sodium phosphate buffer containing 2.5 mM CaCl$_2$ and 35 mM KCl, pH 8.0) in the presence of 1.5 enzymatic units of PLA$_2$ (from *Naja naja* venom) and incubated at
room temperature with gentle stirring. Aliquots were taken at different times to obtain different degrees of phospholipid degradation. The reaction was stopped by a 10-fold dilution with cold phosphate buffer (10 mM sodium phosphate buffer containing 3 mM EGTA, pH 7.4). Finally, the samples were centrifuged at 100,000 × g for 45 min. The resulting pellet was resuspended in buffer A and kept at −60 °C until use (26, 41).

**Generalized Polarization (GP) Determination**—After AChR-rich membrane incubation with the exogenous hydrophobic compound, Laurdan was added to give a final probe concentration of 0.6 μM and incubated in the dark for 1 h (36, 42). GP values, obtained from emission spectra obtained with an excitation wavelength of 290 and 360 nm for Förster-type resonance energy transfer (FRET) or direct excitation, respectively, were calculated as GP = (I_{434} – I_{490})/(I_{434} + I_{490}), where I_{434} and I_{490} are the emission intensities at the characteristic wavelength of the gel and liquid-crystalline phases, respectively (43, 44).

**Affinity Column Preparation and AChR Purification**—*T. californica* crude membranes (2 mg/ml protein concentration) were solubilized in 1% sodium cholate for 45 min and then centrifuged for 1 h at 74,000 × g to discard the insoluble material. The AChR was purified by affinity chromatography in the presence of synthetic lipids (45, 46). Briefly, the affinity column was prepared by coupling cystamine to Affi-Gel 10, reduction with dithiothreitol, and final modification with bromoacetylcholine bromide. The supernatant was applied to the affinity column. To facilitate complete exchange of endogenous for defined lipids, the column was then washed with a linear gradient of defined lipids (POPC:POPA:cholesterol 3:1:1; where POPC is palmitoyl-oleoyl phosphatidylcholine, and POPA is palmitoyl-oleoyl phosphatidic acid) dissolved in dialysis buffer (100 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, 0.02% NaN₃, pH 7.8) and containing 1% cholate, from 1.3 to 3.2 mM and then to 0.13 mM lipid concentration (45). The AChR was then eluted from the column with a 0.13 mM lipid solution in 250 mM NaCl, 0.1 mM EDTA, 0.02% NaN₃, 5 mM phosphate, pH 7.8, with 0.5% cholate and 10 mM carbamoylmethylcarbamide (Carb). After elution from the column, the AChR was dialyzed against 1 liter of dialysis buffer with five buffer changes (every 12 h) at 4 °C. The AChR purification was checked by SDS-PAGE, and protein concentration was determined by the method of Lowry (47). The samples were stored at −70 °C until use.

**N-(1-Pyrenyl)maleimide AChR Labeling**—The labeling of TM AChR cysteines was performed according to Li et al. (35) with slight modifications. Briefly, purified AChR (1 mg/ml) solubilized in 1% cholate was incubated in the presence of N-PyRM final concentration of 1 mM for 1 h at room temperature with gentle shaking. The solution was centrifuged for 45 min at 70,000 × g to pellet aggregates. The supernatant was dialyzed against 1 liter of dialysis buffer with five buffer changes (every 12 h) at 4 °C and stored at −70 °C until use. N-PyrM-AChR samples were submitted to SDS-PAGE, and the N-PyrM label was visualized under a UV transilluminator showing that the band corresponding to the γ-subunit was predominantly labeled, as shown in previous work (35).

**Fluorescence Quenching with Acrylamide and Spin-label Free Fatty Acid**—Quenching experiments were developed with N-PyrM-AChR. Excitation wavelength was 345 nm, and the fluorescence emission scanning was from 360 nm up to 500 nm. The maximum pyrene fluorescence emission was recorded at 374 nm. To confirm that the pyrene label was at the TM segment of the AChR, a sample of N-PyrM-AChR was titrated with acrylamide, a water-soluble quencher, up to a concentration of 500 mM. To corroborate the correct quenching process, the intrinsic Trp emission was recorded at 330 nm on the same samples (290-nm excitation wavelength). N-PyrM-AChR samples (20 μg/ml) were first titrated with steroid (HC) or different FFA up to 600 or 60 μM, respectively, and control samples were obtained with the addition of only the vehicle of the exogenous compounds. For each sample, sequential additions of 5-SLFA dissolved in ethanol were performed. After each addition, samples were kept for 30 min at 25 °C before the fluorescence measurement to allow equilibration of the 5-SLFA with the membrane. From the fluorescence data, Stern-Volmer plots were obtained according to the equation F/F₀ = 1 + Ksv[Q], where F₀ and F correspond to the fluorescence emission of pyrene-labeled AChR in the absence and presence of 5-SLFA, respectively, [Q] is the concentration of the quencher, and Ksv is the Stern-Volmer constant (49). Ksv is a measure of the quencher concentration in the vicinity of the fluorophore, which allowed us to obtain topological information on the transverse location of the labeled cysteine.

**RESULTS**

Aminotriarylmethane dyes share structural similarities with many known NCA of the AChR: (a) the presence of aromatic groups, (b) tertiary or quaternary amines and (c) a net positive charge. Lurtz and Pedersen (40) demonstrated that CrV, one such compound, displays higher affinity for the D conformation (in the presence of agonist) than for the R conformation (in the absence of agonist) of the AChR. We took advantage of these differences in affinity to monitor AChR conformational states in the presence of two types of low affinity NCA of the AChR; that is, steroids and FFA. These molecules share the characteristic of being highly hydrophobic compounds. We recently demonstrated that these lipids exert their action on the TM domain of the AChR (26), but the mechanism by which they cause AChR inhibition is not yet known.

**Steroids Induce a Conformational Change of the Resting to the Desensitized AChR State**—To study whether the presence of steroids affects the AChR conformational equilibrium, AChR-rich membranes from *T. californica* were first incubated with increasing concentrations (100 – 600 μM) of steroid (cortisone or HC). Carb was subsequently added to a subset of these membranes, and then all samples were titrated with CrV (Fig. 1a). Remarkably, in the absence of agonist, the curves furnished in the presence of steroid were displaced toward those in the D state. In the presence of agonist, the curves obtained with or without steroid (control) were similar. In the absence of agonist, HC produced a concentration-dependent diminution of the KD of the R state, which approached that of the (control) KD of the D state (Fig. 1b).

**Free Fatty Acids Modify Both the Resting and the Desensitized State of the AChR**—We next studied the effect of different exogenous FFA. AChR-rich membranes from *T. californica* were incubated with increasing concentrations of different cis-un-
saturated FFA (cis-FFA) (arachidonic acid, cis-20:4; linolenic acid, cis-18:3; oleic acid, cis-18:1) followed by the addition of 1 mM Carb to a subset of samples (Fig. 2a). A diminution of the \(K_D\) of CrV was observed when the membranes were treated with cis-18:1 in the absence of agonist. A statistically significant increase in the value of \(K_D\) was observed in the presence of agonist. Similar results were obtained for cis-20:4 and cis-18:3 (data not shown).

Experiments were also performed with saturated FFA (sFFA). AChR-rich membranes from \(T.\) californica were incubated in the presence of increasing concentrations of arachidic acid (20:0) or stearic acid (18:0). In contrast to what was observed with cis-FFA (Fig. 2a), the \(K_D\) obtained showed no statistically significant changes either in the absence or presence of agonist (Fig. 2b). A third structural class of FFA, with trans isomerism, was also tested (Fig. 2c); elaidic acid (trans-18:1).

The observed effects may be due to differential partitioning of the fatty acids into the membrane and not to the chemical properties of the different acyl chains. The magnitude of the incorporation of different FFA to AChR-rich membranes was extensively characterized in a previous work from our laboratory using the fluorescence probe ADIFAB (acyrlyodan-derivatized intestinal fatty acid binding protein) (50). The calculated partition coefficient values allowed us to classify the FFA into three different groups; 1) highly hydrophobic FFA (such as 20:0 and 18:0), 2) less hydrophobic FFA (such as cis-18:1 and trans-18:1), and 3) more hydrophilic FFA (such as 18:0, 18:2, 18:3, 20:4, and 22:6). Here we used FFA from the three groups (20:0, cis and trans 18:1, cis-18:3, and cis-20:4), and the observed effects show no relation with the degree of FFA partition, since the most hydrophobic FFA (i.e. the one exhibiting the highest membrane partition coefficient) did not display any effect, and the two FFA with similar partition coefficients (cis and trans 18:1) showed very different effects.

**Free Fatty Acids and Steroids Together Modify Both Conformational States of the AChR**—The combined effects of FFA and steroids were studied next. Toward this end steroid (HC) or FFA (oleic acid) were added to AChR-rich membranes from \(T.\) californica at saturating concentrations (cf. Figs. 1 and 2), and the second lipid was added subsequently, also at saturating concentration (Fig. 3). The order of addition of the two types of lipids did not matter; either combination resulted in similar final states of the AChR both in the presence or absence of agonist. Furthermore, when CrV was added in the presence of agonist, the \(K_D\) values obtained from samples treated with both HC and oleic acid were similar to those obtained with oleic acid alone.

**Membrane Polarity Modulates the Transition of the AChR to the D State**—To study whether there is a link between AChR conformational state and the physical state of its lipid microenvironment, we measured the physical properties of the membrane in the presence of steroid or FFA. AChR-rich membranes from \(T.\) californica were first incubated with 60 \(\mu\)M FFA or 600 \(\mu\)M HC and then labeled with the polarity-
sensitive fluorescence probe Laurdan. The physical origin of Laurdan spectral properties resides in its capacity to sense the polarity and the molecular dynamics in its environment because of the effect of dipolar relaxation processes (43, 44). The principal dipoles sensed by Laurdan in the membrane are water molecules (51). Thus, GP values depend on the extent of water penetration allowed by the local membrane packing, and in this sense Laurdan GP is extensively used to sense changes in phospholipid order (52). GP values were calculated from the Laurdan emission spectrum, obtained by direct excitation or FRET using the intrinsic fluorescence of the protein as the donor, and are informative of the membrane order in the bulk membrane or in the immediate environment of the AChR protein (Table 1). cis-FFA (oleic acid) decreased GP (i.e. increased the polarity) of both the bulk membrane (direct excitation) and the AChR-lipid microenvironment (FRET excitation), whereas trans-FFA (elaidic acid) and HC did not change the measured physical property of the membranes; sFFA produced a slight increase in membrane polarity. We also measured the polarity of membranes treated with HC plus oleic acid, reproducing the conditions of Fig. 3. Independently of the order of incorporation, both conditions showed the same membrane polarity, similar to that of membranes treated only with oleic acid. cis-FFA, the only exogenous lipid tested that increased membrane polarity/order, perturbs not only the R but also the D state, which is suggestive of a connection between the physical state of the membrane and the ability of the AChR to reach certain conformational states.

In another series of experiments the physical state of the membrane was modified by changing the temperature before CrV titration (Table 1). At 42 °C, AChR-rich membranes from T. californica showed an augmentation of the membrane polarity together with an important increment of the $K_D$ of CrV in the presence of agonist with respect to membranes at 25 °C. Thus, when membrane polarity is perturbed (either by the exogenous lipids or temperature increase) the AChR does not undergo the agonist-driven conformational transition to the D state. In contrast, the R state of AChR appears not to be affected by membrane polarity, indicating that the membrane physical state has no direct influence on the R state of the AChR. A more detailed study of the $K_D$ of CrV as a function of temperature showed that as the temperature increased, the $K_D$ of CrV in the presence of agonist also increased (Fig. 4). The extent of this increment was found to be proportional to the extent of the change in membrane order (higher temperatures or higher cis-FFA concentrations are directly proportional to higher membrane physical alterations and inhibit more pronouncedly the ability of the AChR to reach to the D state). On the other hand, in the absence of agonist, the $K_D$ of CrV remained constant, confirming the view that a certain degree of membrane polarity is necessary for the AChR to reach the D state but not to maintain the R state.

AChR-rich membranes from T. californica were incubated in the presence of PLA$_2$ for different periods to produce increasing concentrations of endogenously released FFA. A subset of these membranes was titrated with CrV, and another subset was labeled with Laurdan to measure membrane polarity (Fig. 5). The $K_D$ of CrV clearly increased as a function of time in the presence of agonist but remained constant in its absence. An increase in GP was observed as a function of hydrolysis time, which correlates well with the increase in the $K_D$ values in the

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### FIGURE 3. Effect on $K_D$ of CrV of the competition between FFA and steroids. T. californica AChR-rich membranes, incubated in the presence of HC (600 µM), cis-18:1 (60 µM), HC (600 µM) plus cis-18:1 (60 µM), and cis-18:1 (60 µM) plus HC (600 µM), were titrated with CrV in the absence or in the presence of 1 mM Carb (closed and open bars, respectively). Each bar corresponds to the average ± S.D. of at least four independent experiments. * Statistically significant differences with respect to control values ($p < 0.001$).

### TABLE 1

$K_D$ of CrV and GP values of Torpedo membranes in the presence of FFA or steroids

| Temperature | Membranes | CrV $K_D$ [nM] | Laurdan GP$^+$ | Direct excitation | FRET |
|-------------|-----------|----------------|----------------|------------------|------|
|             |           | $-\text{Agonist}$ | $+\text{Agonist}$ |                   |      |
| 25 °C       | Control   | 378.6 ± 83.1    | 40.5 ± 11.2     | 0.37 ± 0.01      | 0.41 ± 0.01 |
|             | cis-18:1 | 198.3 ± 64.9$^b$ | 157.7 ± 43.5$^a$ | 0.18 ± 0.01$^b$ | 0.24 ± 0.00$^a$ |
|             | trans-18:1| 184.5 ± 46.5    | 165.7 ± 23.5    | 0.54 ± 0.01$^a$ | 0.39 ± 0.00$^b$ |
|             | +20:0     | 200.5 ± 60.5    | 209.2 ± 50.5    | 0.58 ± 0.00$^b$ | 0.41 ± 0.00$^a$ |
|             | +HC       | 186.5 ± 56.6$^a$ | 31.6 ± 5.5      | 0.36 ± 0.01$^b$ | 0.41 ± 0.00$^a$ |
|             | cis-18:1 + HC | 198.5 ± 2.7$^b$ | 118.4 ± 22.6$^a$ | 0.17 ± 0.01$^b$ | 0.23 ± 0.01$^a$ |
|             | +HC + cis-18:1 | 234.9 ± 84.5$^a$ | 38.9 ± 8.5$^a$ | 0.10 ± 0.01$^b$ | 0.17 ± 0.01$^a$ |

| 42 °C       | Control   | 375.9 ± 93.4    | 247.2 ± 75.4    | 0.37 ± 0.01      | 0.41 ± 0.01 |

$^a$ GP values vary from 0.6 to 0.3 (from pure gel phase to pure liquid-crystalline phase).

$^b$ Statistically significant differences with respect to control values ($p < 0.001$).
presence of agonist. Thus, the $K_D$ in the presence of agonist is sensitive to either a decrease or an increase of the membrane polarity, which implies that the AChR needs a proper fluidity to reach the D state.

**AChR Conformational Changes Induced by the Presence of FFA and HC**—To obtain complementary evidence on AChR conformational changes caused by FFA or HC, we performed a quenching experiment with purified AChR from _Torpedo_ membrane labeled with a fluorescent lipophilic probe specific for sulfhydryl groups in a hydrophobic environment following the protocol used by Narayanaswami et al. (35). These authors studied the spatial disposition of the AChR-bound pyrene with respect to the membrane bilayer by quenching the pyrene fluorescence with spin-labeled lipid probes. They concluded that the pyrene fluorophore is located in a shallow position, close to the membrane-water interface rather than in the bilayer hydrophobic core. Here we studied whether the addition of FFA or HC modified the position of the fluorescent tag inside the bilayer, an indication of protein TM movement.

To verify that the pyrene probe was indeed inserted into the hydrophobic region of the bilayer, we used the effective water-soluble quencher, acrylamide (53). The fluorescence intensity of pyrene (excitation at 345 nm) did not decrease upon the addition of acrylamide, whereas AChR intrinsic fluorescence (excitation at 290 nm) was effectively quenched (60% of the AChR Trp residues are exposed to the extramembranous region) (Fig. 6a). Spin-labeled stearic acid with the nitroxide group attached to carbon 5 (5-SLFA) was added to membranes previously treated with different FFA or HC. The quenching profile of the pyrene fluorescence in each case and the calculated Stern-Volmer quenching constants ($K_{SV}$) are shown in Fig. 6, b and c, respectively. These results indicate that the TM distribution of the label did not change when the membranes were treated with _trans_-FFA and _s_FFA but did change with _cis_-FFA and HC, suggesting changes in AChR TM topography that do not depend on the membrane physical state (_cis_-FFA increases membrane fluidity whereas HC does not, Table 1).

**DISCUSSION**

In the present work we seek to study the conformational changes of the AChR caused by steroids and FFA, two classes of highly hydrophobic molecules. Previous studies indicated that these molecules act as NCA of the AChR and that their effects occur inside the membrane (4–6, 9–11, 15, 54). Recently, we found that these two types of lipid share common sites at the AChR TM region (26). However, the link between the presence of these molecules in the bilayer and the functional inhibition of the AChR remains elusive. As described in the Introduction, the functionality of the AChR (both the passage of ions across the pore...
R state and also precluded it from reaching the agonist-induced D state.

The key difference between the different FFA studied here resides in the double bond. cis isomerism produces a kink of the acyl chain whereas trans isomerism does not; trans isomers exhibit a spatial arrangement similar to that of sFFA (57). Consequently, cis-FFA produce an increase in AChR rich-membrane polarity measured with the fluorescent probe Laurdan (Table 1 and Ref. 50). GP values vary from 0.6 to 0.3 in pure model systems (from pure gel phase to pure liquid-crystalline phase); however, in biological membranes they show a narrower range of values because of their molecular composition. In this sense the GP variations caused by the presence of cis-FFA, PLA₂ treatment or temperature increment, are large (for comparison, see Refs. 58–61). Laurdan possesses an exquisite sensitivity to the phase state of the membrane, having the capacity to sense the polarity and the molecular dynamics of dipoles in its environment due to the effect of dipolar relaxation processes (43, 44). An advantage of measuring membrane polarity with Laurdan is the possibility of obtaining dynamic information from steady-state measurements. However, to obtain a further characterization of the and the transition from the R to the D states of the receptor) is highly sensitive to the composition of the surrounding lipid. Speculative models on the role of membrane fluidity on AChR function have been proposed (55, 56). Baenziger and co-workers suggest that membrane fluidity modulates the relative proportion of AChR in the R and D states (45).

In the present work we took advantage of the different affinities that the fluorescence dye CrV displays for the R and D states of the AChR. As expected, a desensitizing concentration of agonist decreased the KD, for CrV, indicating that the AChR undergoes conformational changes leading to the high affinity D state, as previously reported by Lurtz and Pedersen (40). Surprisingly, when AChR-rich membranes were incubated with steroids (HC or cortisone) in the absence of agonist, KD values decreased, approaching those observed for the agonist-induced D state. This result clearly indicates that steroids induce a conformational change of the AChR.

FFA exert different effects on the AChR conformational state depending on their structural characteristics. As shown in Fig. 2, of all FFA tested, only cis-FFA drove the AChR out of the membrane physical state, other measurements must be undertaken (for example, steady-state and time-resolved anisotropy).

The lack of transition to the D state of the AChR upon cis-FFA addition can be mimicked by raising the temperature, which disorders the membrane bilayer (Fig. 4), or by treatment with PLA₂, which decreases the polarity of the membrane. The main effect was observed in the presence of agonist; the KD increased significantly, which is a clear indication that the AChR did not shift to the D state (Fig. 5).

Ligand binding regulates the conformational paths of AChR opening and desensitization, possibly through several intermediate states (see e.g. Ref. 62). In this study we focused mainly on the R and the D states, which represent the initial and final states of a series of conformations of the AChR channel protein that lead from a nonconductive state to a final refractive nonconductive state, passing through a transient conductive state. This is summarized in the sequential linear model of the AChR activation equilibrium (63–65) (Scheme 1), where R is the AChR in the R state; A the agonist; RA, RA₂, RA₂*, and RA₂ represent the AChR with one agonist molecule, with two ago-
perturbation of AChR Conformational State

Perturbation of AChR Conformational State

k_1  k_2  \beta
R + 2A \rightleftharpoons RA + A \rightleftharpoons RA_2 \rightleftharpoons RA_2^* \rightleftharpoons RA_2^D
k_1  k_2  \alpha

SCHEME 1.

R + 2A \rightleftharpoons AR + A \rightleftharpoons A_2R \rightleftharpoons A_2R^*
/
/
/
/

D + 2A \rightleftharpoons AD + A \rightleftharpoons A_2D \rightleftharpoons A_2D^*

SCHEME 2.

nist molecules, with two agonist molecules in a transient active open configuration, and with two agonist molecules in a refractory closed D state, respectively; \( k_1 \) and \( k_2 \) are the association constants; \( k_{-1} \) and \( k_{-2} \) are the dissociation constants; \( \beta \) and \( \alpha \) are the opening and closing rate constants, respectively. Many endogenous and exogenous conditions can modify this allosteric equilibrium (e.g. single-point mutations and exposure to some drugs). Dilger and Liu (66) considered that each state of channel activation has a corresponding desensitized state as summarized (Scheme 2), where D, AD, and A_2D^* correspond to the same states as in Scheme 1 but with the AChR in different desensitized non-conductive states. In the present work, the D state tested with CrV should correspond to the A_2D^*. Ryan et al. (67), using infrared differential spectroscopy, showed that different portions of the AChR interconvert between the R and D states independently of one another and that the binding of NCA may lead to an intermediate conformational structure between both states. Our data indicate that the mere presence of FFA or steroids drives the AChR out of the R state but that in all cases the AChR falls short of reaching the full D state, probably remaining in one of the intermediate D states.

Both FFA and steroids affect the AChR-rich membrane polarity differently; whereas cis-FFA increase membrane polarity, trans-FFA, sFFA, and steroids cause practically no changes in membrane polarity (see GP values in Table 1). The observation of similar effects on AChR conformational states (cis-FFA and steroids), despite different membrane physical states, indicates that the presence of hydrophobic molecules at the lipid-protein interface is responsible for the induction of conformational changes in the AChR. It is probable that direct contacts between these exogenous molecules and TM portions of the AChR drive the receptor out of the R state and, hence, inhibit its function.

Structural studies (68), confirming previous affinity labeling studies (69, 70), showed that M4 TM segments make extensive contact with the lipid bilayer (27). Although M4 is relatively far apart from both the channel lumen and the transmitter binding sites, functional analyses have shown that mutations of some of the residues in the M4 segment of different AChR subunits alter the gating equilibrium constant (15, 71–74). These results suggest that these M4 residues experience a motion during the gating reaction. Mitra et al. (75), working with 88 different AChR constructs having mutations of 21 different M4 residues in all five subunits, demonstrated that the M4 segments move synchronously near the middle of the gating reaction, following the movement of \( \alpha \)-subunit extracellular domains and M2-M3 linkers but before the motion of the \( \delta \)-subunit M2 segment. Auerbach (76) visualized the AChR gating as "a sequence of back-and-forth motions of a small number of rigid-body domains rather than as a continuous, smooth transition joining the stable close and open states," speculating that the dynamics of AChR gating is governed by the Brownian motion of its discrete moving parts. The presence of molecules such as FFA or steroids at the lipid-AChR interface may alter lipid-protein interactions in such a way that TM rigid-body domains contribute to the adoption of the AChR desensitized state, as indicated by fluorescence quenching experiments with purified AChR from Torpedo membrane labeled with N-PyrM in the presence of steroids and cis-FFA (Fig. 6). Because the perturbation inside the membrane is permanent (presence of exogenous molecules), once the AChR conformation is shifted to the D state, it cannot return to the R state.

cis-FFA caused a second effect on AChR-rich membranes, preventing the AChR from reaching the D state. Because different experimental conditions associated with changes in the membrane physical state (presence of exogenous cis-FFA, PL_A treatment of AChR-rich membranes, or temperature increase) tend to modify the closed \( \Leftrightarrow \) open \( \Leftrightarrow \) desensitized equilibrium in a similar way, this second effect seems not to depend on the presence of specific molecules at the lipid-AChR interface but rather to be unspecific, mainly associated to changes in the membrane physical state. Hampering the R to D transition probably results in an increment of AChR function. Temperature has been shown to have a greater influence on the rate of conformational changes associated with the transitions between open and closed states in the AChR than on the ion conductance process (77).

The distinct effects that exogenous molecules and changes in the membrane physical state cause on AChR conformation can be analyzed in light of recent proposals that envision the activation and desensitization gates of the AChR acting as distinct molecular entities and involving the rearrangement of different portions of the molecule (78). Thus, whereas foreign molecules at the lipid-protein interface probably modify the "activation gate," leading to an intermediate D state, membrane physical state changes (in particular changes in membrane order or polarity) are likely to perturb the "desensitization gate." The sequential addition of steroids and cis-FFA verify this hypothesis; steroid alone, in the absence of agonist, drove the AChR out of its R state but did not affect the D state in the presence of agonist. The subsequent addition of a cis-FFA to steroid-treated AChR in the absence of agonist caused no further changes in AChR conformation, whereas in the presence of agonist the AChR was precluded from reaching the D state, which can be induced by cis-FFA alone (Fig. 4).

The present work is a step forward in our understanding of the mechanisms of action of low affinity NCA of the AChR. Hypothetically, the direct contact between these exogenous molecules and the AChR TM segments might remove the AChR from its R state, causing its functional inhibition. Furthermore, the relationship between the membrane physical state and the AChR conformational state identified in the present work might explain why the AChR activation equilibrium is affected by changes in its lipid microenvironment.
