Conformation-Sensitive Steroid and Fatty Acid Sites in the Transmembrane Domain of the Nicotinic Acetylcholine Receptor†

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Received July 10, 2006; Revised Manuscript Received December 20, 2006

ABSTRACT: The mechanism by which some hydrophobic molecules such as steroids and free fatty acids (FFA) act as noncompetitive inhibitors of the nicotinic acetylcholine receptor (AChR) is still not known. In the present work, we employ Förster resonance energy transfer (FRET) between the intrinsic fluorescence of membrane-bound *Torpedo californica* AChR and the fluorescent probe Laurdan using the decrease in FRET efficiency (*E*) caused by steroids and FFA to identify potential sites of these hydrophobic molecules. Structurally different steroids produced similar changes (ΔE) in FRET, and competition studies between them demonstrate that they occupy the same site(s). They also share their binding site(s) with FFA. Furthermore, the FRET conditions define the location of the sites at the lipid−protein interface. Endogenous production of FFA by controlled phospholipase A_2 enzymatic digestion of membrane phospholipids yielded ΔE values similar to those obtained by addition of exogenous ligand. This finding, together with the preservation of the sites in membranes subjected to controlled proteolysis of the extracellular AChR moiety with membrane-impermeable proteinase K, further refines the topology of the sites at the AChR transmembrane domain. Agonist-induced desensitization resulted in the masking of the sites observed in the absence of agonist, thus demonstrating the conformational sensitivity of FFA and steroid sites in the AChR.

The nicotinic acetylcholine receptor (AChR),[1] the best characterized member of the ligand-gated ion channels superfamily, is an integral membrane protein composed of five homologous subunits organized pseudo-symmetrically around a central pore (*1*). Each subunit contains a relatively large extracellular domain, four hydrophobic transmembrane (TM) segments (M1−M4) connected by loops of varying length and ends with a short extracellular carboxyl terminal domain. Three concentric rings can be distinguished in the AChR TM region (*2*). 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 microenvironment (*2*, *3*).

The binding of two agonist molecules triggers a conformational change that brings about a brief channel opening allowing the flux of ions across the membrane. Several compounds inhibit the AChR-mediated ion permeation function, some acting competitively at the binding site(s) and others using a noncompetitive mechanism. It is accepted that high affinity noncompetitive inhibitors (NCI) exert their effect through the blockage of the ion channel proper, whereas the mechanism involved in the action of some low affinity NCI has not yet been elucidated. The AChR−lipid interface has been the focus of great interest as the potential site of action of a group of low-affinity NCI that share the property of being highly hydrophobic (for a review see ref *3*).

Steroids constitute a subgroup of low-affinity NCI. Blanton et al. (*4*) demonstrated by photolabeling assays that the steroid promegestone acts at the lipid−protein interface. Nurowska and Ruzzier (*5*) interpreted that the binding site of the steroid hydrocortisone is located at the AChR extracellular portion, near the agonist-binding site, but no physical evidence of this site was provided in their study. Free fatty acids (FFA) also inhibit the AChR in a noncompetitive manner (*6*−*8*), and it has been suggested that they cause AChR inhibition by acting on an allosteric site (*9*). It has also been demonstrated that FFA display high affinity for the lipid−AChR interface (*10*−*13*). The relative affinity of FFA for the native membrane-bound AChR has been determined to be the highest among all lipids studied to date (*13*), as is also the case for spin-labeled androstane (*14*). Distinct sites for FFA have been characterized both in model systems (*15*) and in native *Torpedo marmorata* membranes

† This work was supported in part by grants from The Academy of Sciences for the Developing World (TWAS) and the Agencia Nacional de Promoción Científica (FONCYT) to S.S.A. and from the Universidad Nacional del Sur, the Consejo Nacional de Investigaciones Científicas y Técnicas and the Agencia Nacional de Promoción Científica (FONCYT) to F.J.B. and NIH 1 R01 DA015389 to R. Lukas in collaboration with F.J.B.

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; AA, arachidonic acid; DOPC, 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycerophosphocholine; chol, cholesterol; FFA, free fatty acids; *E*, fluorescence energy transfer efficiency; FRET, Förster resonance energy transfer; LPC, lisophosphatidylcholine; NCI, non-competitive inhibitors; PMSF, phenylmethanesulfonyl fluoride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2; SM, sphingomyelin; TLC, thin layer chromatography; TM, transmembrane; Trp, tryptophan.

10.1021/bi061388z CCC: $37.00
Published on Web 02/24/2007

(*16*, *17*). In the latter case, we could discriminate between annular sites for FFA and phospholipids and nonannular sites for FFA and cholesterol. The first shell of lipids surrounding the AChR protein is termed "annular lipid" and purported to be occupied mainly by phospholipids (*18*). Lipid molecules at annular sites interact with the protein in a relatively less specific manner, and the rate of exchange between annular shell lipid and the bulk membrane lipid is fast (see ref 3). On the basis of competition studies, "nonannular lipid" has been associated with binding sites to which cholesterol is bound but phospholipids are not (*15*).

Two hypotheses have been proposed to explain the mechanism of action of NCI on the AChR: (a) an indirect mechanism, in which the presence of the exogenous compound in the AChR microenvironment changes the biophysical properties of the membrane and leads to a conformational change of the AChR protein (*19*, *20*); and (b) a direct mechanism of action, involving the interaction of the exogenous compound with the AChR, either by inducing a conformational change or by displacing an essential lipid required for proper gating of the ion channel (e.g., negatively charged phospholipids and/or cholesterol (*20*−*23*)).

In the present work, we use fluorescence spectroscopy to study the localization of possible sites for three structurally different steroids (cortisone (cort), hydrocortisone (HC), and 11-hydroxy-progesterone (11-OH-P)) and their relation to sites for FFA. We measure the variations (ΔE) in energy transfer efficiency (E) between the intrinsic AChR fluorescence (donor) and the fluorescent probe Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) (acceptor) (*16*, *24*) caused by the presence of exogenous steroids and/or FFA. The results indicate that the three steroids assayed, although structurally different, are localized at the AChR−lipid microenvironment and share the sites with the FFA. Furthermore, agonist-induced desensitization resulted in ΔE values different from those caused by steroids or FFA alone, thus indicating that steroid and FFA sites are sensitive to the conformational state of the AChR protein (at least between resting and desensitized conformers). Controlled proteolysis of the AChR extracellular moiety by proteinase K digestion further indicates that FFA and steroid sites are located at the TM domain, an observation that is reinforced by another series of experiments using phospholipase A_2-mediated release of endogenous FFA, in which case a ΔE profile similar to the one obtained with exogenous FFA was observed.

EXPERIMENTAL PROCEDURES

Materials. Torpedo californica specimens obtained from the Pacific coast of California (USA) were killed by pithing, and the electric organs were dissected and stored at −70 °C until further use. Laurdan was purchased from Molecular Probes (Eugene, OR). Proteinase K was from Promega (Madison, WI). All the others drugs were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods. Preparation of AChR-Rich Membranes. Membrane fragments rich in AChR were prepared from the electric tissue of *T. californica* as described previously (*25*). Specific activities on the order of 2.0−2.8 nmol of α-bungarotoxin sites/mg of protein were obtained. The orientation of AChR in the membrane vesicles was determined as described by Hartig and Raftery (*26*) 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 a previous work from our laboratory (*27*).

For the fluorescence 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 of protein/mL (0.2 μM). The optical density of the membrane suspension was kept below 0.1 to minimize light scattering.

Preparation of Steroid and Arachidonic Acid Solutions. Cort and HC were dissolved in (1:1) ethanol/DMSO; 11-OH-P was dissolved in (1:2) ethanol/DMSO, and arachidonic acid (AA) was 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 for 30 min at 22 °C before the actual fluorescence measurement to allow equilibration of the hydrophobic compounds with the membrane preparation. Although the steroids were studied at concentrations up to 900 μM, the free steroid concentration in the aqueous phase was very low because of the high partition coefficient of the ligand in the membrane phase.

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 2 mL quartz cuvettes. Emission spectra were corrected for wavelength-dependent distortions. The temperature was set at 22 °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% all along the experiments. The samples were incubated in the dark for 60 min at room temperature. When the experiments were performed on desensitized AChR, 1 mM carbamoylcholine (Carb) was added 15 min before addition of Laurdan.

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 (*28*) E can be calculated as follows:

$$E = 1 - (I/I_D) \tag{1}$$

where I and I_D are the emission intensities in the presence and in the absence of the acceptor, respectively. Here, I corresponds to the maximal intrinsic protein emission intensity, which is 330 nm. The excitation wavelength was set at 290 nm.

Steroid molecules absorb at the Trp excitation/emission wavelengths; hence, the steroid inner filter effect was corrected according to the following formalism:

$$I_{corr} = I \,\text{antilog}[(OD_{290} + OD_{330})/2] \tag{2}$$

where I is the measured fluorescence intensity of the Trp (330 nm) excited at 290 nm, and OD_{290} and OD_{330} are the

optical densities of the sample at the excitation and emission wavelength, respectively (*29*).

For each condition, the normalized FRET efficiency was calculated as follows:

$$E_{nor} = (I - I^{Lau})/(I_o - I_o^{Lau}) \quad (3)$$

where I_o^{Lau} and I_o values are the initial emission intensities of the samples with or without Laurdan for any given condition, respectively.

Enzymatic Digestion of Extramembranous Regions of the AChR was performed as in Görne-Tschelnokow et al. (*30*) with slight modifications. AChR-rich membranes were suspended at a final concentration of 1 mg/mL in phosphate buffer (10 mM sodium phosphate buffer, 1 mM $CaCl_2$, pH 8.0) in the presence of 1 mg/mL proteinase K and incubated at 37 °C with gentle shaking. Aliquots were taken at different times to obtain increasing proportions of extracellular AChR digestion. Proteinase K digestion was stopped by addition of 1 mM PMSF and 0.5 M NaCl. The samples were shaken for 60 min on ice to remove peptide aggregates, and then diluted 10-fold with cold 10 mM sodium phosphate buffer, pH 8.0. Finally, the samples were centrifuged at 100000*g* for 45 min. The resulting pellet was resuspended in buffer A. Protein concentration in each sample was measured by the method of Lowry (*31*), and the percentage of degradation was calculated with respect to undigested control samples (100% recovery or 0% degradation) devoid of proteinase K.

To maintain the same amount of AChR per sample in all cuvette experiments, such that the number of AChR transmembrane portions remained constant, each proteolysis condition had the same initial amount of protein. At the end of the experiment, the amount of protein was compared with that in the control sample to account for any loss due to sample manipulation.

Endogenous FFA Production by Phospholipase A_2 (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 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 100000*g* for 45 min. The resulting pellet was resuspended in buffer A. To verify the effectiveness of the PLA_2 reaction, lipids were extracted from each sample according to the procedure of Bligh & Dyer (*32*) and resolved into classes by thin layer chromatography (TLC) on commercial plates as follows. The samples were dried under a stream of N_2; chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5, per vol) was used as the first developing solvent up to the middle of the TLC plate, followed by hexane/ether/acetic acid (60:40:2, per vol) up to the top. Comparison of the different samples clearly shows the increment in lysophospholipids and FFA and, conversely, the decrease in phospholipids as a result of PLA_2 action (Supporting Information).

Data Analysis. Regression analysis was performed with the null hypothesis that the overall slope (β) is zero, considering a p value of less than 0.05 for rejection. Intergroup comparisons were carried out using the paired Student's t-test.

RESULTS

Different Steroids Share a Common Site. To study AChR−steroid interactions, we used the AChR−Laurdan FRET pair (the intrinsic fluorescence of the *T. californica* AChR as the donor and that of the extrinsic fluorescent probe Laurdan as the acceptor) as in previous studies from our laboratory (*16*−*17*, *24*). Changes in FRET efficiency (ΔE) provided a measurement of the displacement of the acceptor molecules (Laurdan) from the AChR microenvironment by the added exogenous molecule. We chose three steroids that were shown in previous work to provoke the strongest inhibition of the AChR (*8*, *33*, *34*): cort, HC, and 11-OH-P. The three steroids assayed produced a concentration-dependent reduction of E with similar profiles (Figure 1). The steroid dose dependence of the E was described by a sigmoidal curve in all cases, suggesting a direct competition between Laurdan and the exogenous ligand. DC_{50} (displacement concentration) values of 367.2, 359.1, and 298.9 μM were obtained for HC, cort, and 11-OH-P, respectively. An apparent saturation was obtained with steroid concentrations below 600 μM. To further analyze the data, a concentration−response curve was obtained for each steroid using the Hill equation to yield a binding parameter (BP) as a function of ligand concentration (insets, Figure 1). The BP was calculated assuming that the decrease in E is proportional to the number of exogenous molecules residing at the lipid−AChR interface, according to the following equation:

$$BP = E^{-1} - 1 \quad (4)$$

Here the inverse of E is used to yield a direct correlation between BP and the amount of liganded molecules. Unity was subtracted from the reciprocal of E to obtain a BP value equal to zero in the absence of ligand. A Hill coefficient greater than 2 was obtained for each steroid ($n = 3.5$ for cort, $n = 3.2$ for 11-OH-P and $n = 2.9$ for HC), which indicates a positively cooperative binding of the three ligands to more than one common site on the AChR oligomer.

To determine whether the similar maximal ΔE values caused by different steroids resulted from binding to the same site(s) independently of their physical and stereochemical characteristics, we conducted an additional series of experiments in which AChR-rich membranes were titrated first with a given steroid (HC, 11-OH-P or cort) up to 600 μM, and subsequently a second steroid was added to the sample. If different steroids bind to topologically different sites, E should decrease further, whereas if the two steroids compete for the same site, ΔE should not change. Figure 2 shows the latter to be the case, since the ΔE obtained with the first steroid remained constant upon addition of a second, different steroid; the same occurred when further additions of the same steroid were made. Thus, structurally different steroids share a common site.

The Steroid Site Is also Shared by FFA. Since steroids and FFA exhibit NCI-like pharmacological effects, the possibility of their being located at similar sites at the lipid−AChR interface was considered. To test this hypothesis, AChR−membrane samples were titrated with the FFA

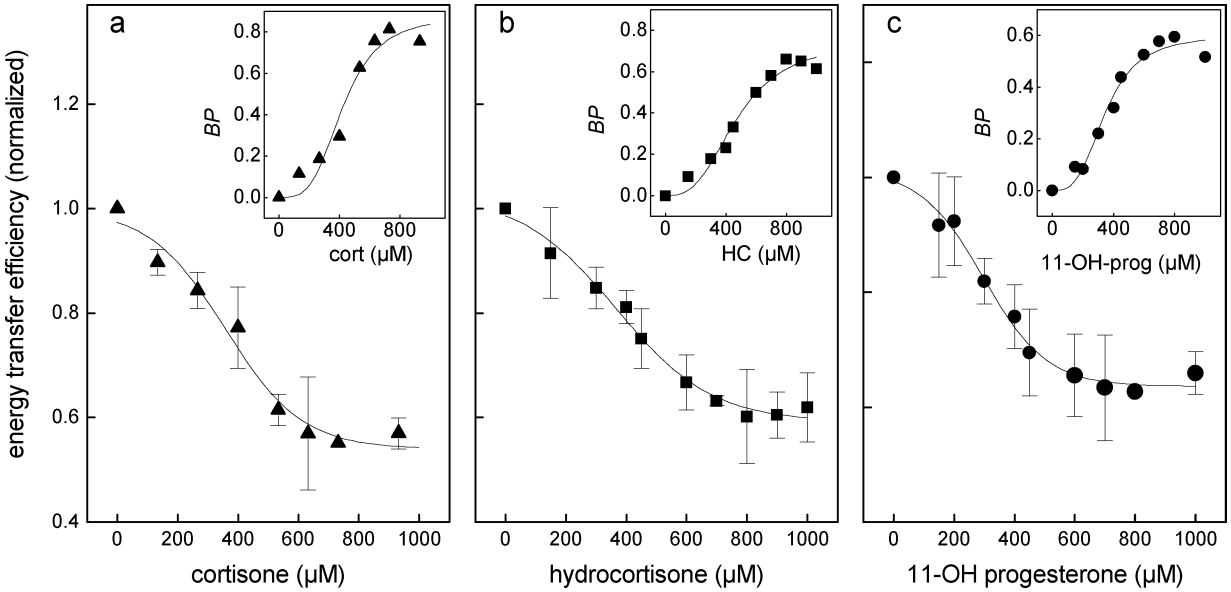

FIGURE 1: (a−c) Changes in normalized FRET efficiency (E) between intrinsic fluorescence in native *T. californica* AChR-rich membrane and Laurdan upon addition of increasing concentrations of the indicated steroids. An initial E value of 0.275 ± 0.018 was obtained in the absence of steroid. The E values obtained in the presence of steroid were normalized with respect to the corresponding E value in its absence (100%). Each point corresponds to the average ±S.D. of more than six independent experiments. See statistical analysis in the text. Inset: binding parameter (BP = $E^{-1} - 1$) calculated as described in the text using the Hill equation.

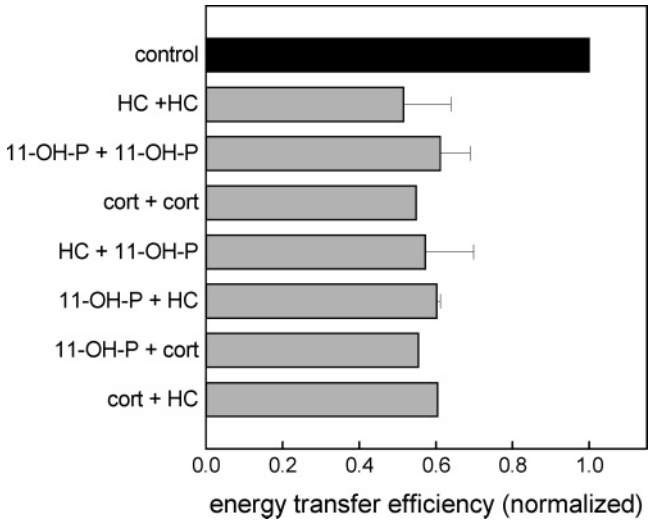

FIGURE 2: E for the AChR/Laurdan pair in *T. californica* rich-AChR membranes. Membranes in the presence of 0.6 μM Laurdan were first saturated with hydrocortisone (HC), 11-hydroxy-progesterone (11-OH-P), or cortisone (cort) up to a concentration of 600 μM. Subsequent additions of the same steroid (up to 300 μM) or a different one were made up to a final steroid concentration of 900 μM. An initial E value of 0.275 ± 0.018 was obtained in the absence of steroid. The control bar corresponds to AChR-rich membranes in the presence of 0.6 μM Laurdan titrated only with solvent. Each bar corresponds to the average ±S.D. of at least four independent experiments. The differences between experimental conditions were not statistically significant.

arachidonic acid (AA) (Figure 3a). The dose−response curve for AA was fitted with a sigmoidal curve with a DC_{50} value of 8.1 μM; apparent saturation was reached at AA concentrations below 20 μM. The FFA data were analyzed using the same formalism that we applied to the steroid data (see above), yielding a dose−response curve in the form of a binding parameter (inset, Figure 3a). As observed with steroids (Figure 1), a Hill coefficient of 2.2 was obtained.

The similarity between the dose−response curves corresponding to AA and the three steroids studied here suggests the occurrence of a common binding site for both types of compounds. To corroborate this hypothesis, we conducted an additional series of experiments in which AA or HC were added up to saturation (20 or 600 μM, respectively) followed by addition of the second ligand. In all cases, the final ΔE values were the same and, more importantly, effects were not additive (Figure 3b).

Topology of the Steroid and FFA Sites in AChR-Rich Membranes. To further dissect the location of the common steroid and FFA site(s), we performed controlled enzymatic hydrolysis of AChR-rich membranes with proteinase K, a membrane-impermeable enzyme. Proteinase K digestion of *Torpedo* AChR-rich membranes has been worked out by Hucho and co-workers (*12*, *30*), and it is currently a widely accepted technique to produce membrane-bound AChR with variable degrees of digestion of the extracellular protein moiety (*35*). The rationale for using proteinase K was to assess whether both FFA and steroid sites were localized at the transmembrane region of the AChR, thus discarding the possibility that extracellular sites were involved. Membrane preparations were grouped according to the remaining membrane-bound AChR protein: 100% (control, without proteinase K), 80−60%, 60−40%, 40−30%, and <30% protein. The amount of TM protein per Laurdan molecule (i.e., the number of putative sites) was kept constant. This was achieved by using as a reference AChR-rich membranes submitted to all steps of the protocol but without proteinase K (0% digestion).

Torpedo AChR has 31 of its 51 Trp residues located in the extracellular region, with only one in the TM region (Trp^{458} in the AChR γ subunit) and the other 19 in the cytoplasmic moiety. The FRET process between the intrinsic fluorescence and Laurdan was maintained up to 30% digestion. Effective FRET was not observed in membrane preparations with <30% of the original protein, possibly

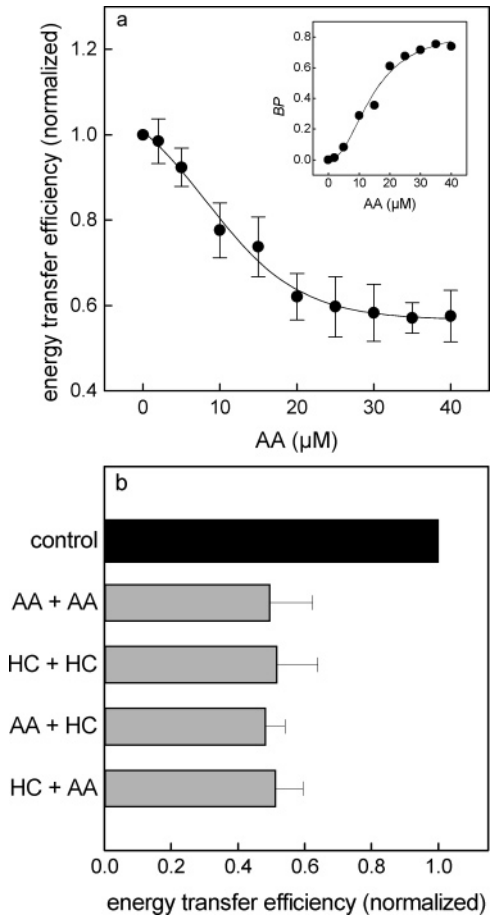

FIGURE 3: *E* for the AChR/Laurdan pair in *T. californica* rich-AChR membranes. (a) *E* as a function of increasing concentrations of AA. The initial *E* value in the absence of AA was 0.282 ± 0.021. Inset: binding parameter (BP = $E^{-1} - 1$) calculated as in Figure 1. (b) Membranes in the presence of 0.6 μM Laurdan were first saturated with AA (20 μM) or HC (600 μM). Subsequent additions of AA or HC were made up to concentrations of 40 μM (AA + AA), 900 μM (HC + HC), or 20 μM of FFA and 600 μM of steroid (AA + HC or HC + AA). The control bar corresponds to AChR-rich membranes in the presence of 0.6 μM Laurdan titrated only with solvent. Each bar corresponds to the average ± S.D. of more than six independent experiments. See statistical analysis in the text.

because the TM portions of the protein disorganize upon extensive digestion of the AChR. This is consistent with the fact that 75% of the AChR mass is extramembranous (*36*).

HC was added to each group of membranes and Δ*E* was measured. Figure 4a shows values of *E* obtained with 600 and 900 μM HC. Δ*E* was similar for all the digested samples, with no statistically significant differences. Experiments performed with 20 μM and 35 μM AA yielded similar results (Figure 4b). This series of experiments indicates that the binding site(s) are preserved as long as the AChR TM region is conserved.

Competition studies were performed next on the proteinase K-digested samples. Studies with 80−60% and 60−40% digested membranes showed results similar to those obtained with native membranes (data not shown). The Δ*E* values resulting from addition of 600 μM HC plus 10 μM AA or additional 200 μM HC were not significantly different. Furthermore, no statistically significant differences were observed between membranes treated with 20 μM AA + 200 μM HC and those exposed to a further addition of 10 μM AA.

Topology of Sites for Endogenously Generated FFA in AChR-Rich Membranes. Membrane samples treated with phospholipase A_2 for different reaction times were used for FRET experiments using Laurdan as acceptor. PLA_2 hydrolyzes phosphatidylcholine and phosphatidylethanolamine, releasing the FFA from the second carbon group of the glycerol backbone to produce lysophosphatidylcholine (see the lipid characterization of the samples in Supporting Information). If the FFA enzymatically generated inside the membrane reach sites at the lipid−protein interface, when Laurdan molecules are added they have to compete with these endogenously produced FFA and hence a lower *E* value should result, which is the case. Figure 5a shows that the *E* value decreased after 1 min, reaching a decrease in Δ*E* of 25% at 5 min and a 27% diminution after 20 min digestion. The decrease in Δ*E* suggests that endogenous FFA also localize at sites at the lipid−protein interface.

An additional experiment was performed to determine whether both endogenous and exogenous FFA share the same sites. Figure 5b shows that whereas the addition of 15 μM AA to the control sample caused a diminution of *E*, as the PLA_2 reaction time increased the Δ*E* caused by the addition of exogenous AA decreased, inducing no diminution at all when the samples were hydrolyzed for 20 min. This can be most economically explained by the fact that as the PLA_2 reaction time increases the amount of endogenously generated FFA also augments, and hence endogenous and exogenous FFA compete for the same sites. As the amount of endogenously generated FFA after 20 min PLA_2 digestion is so high (Supporting Information), subsequent addition of exogenous FFA (AA) cannot produce any further diminution of *E*.

Driving the AChR to the Desensitized Conformation Modifies the Availability of the Sites. The AChR undergoes conformational changes between resting-activable (R) and desensitized-inactive (D) states. The effect of the addition of AA or HC on the Δ*E* was studied on R and D (agonist-induced, 1 mM Carb) states of *T. californica* AChR (Figure 6). In the case of desensitized AChR-rich membrane preparations, no differences were apparent between HC and AA treatments, indicating that the two hydrophobic molecules exert similar effects, as was also the case with membranes in the R state (see Figures 1 and 3). However, when the AChR was in the D state, the steroid or AA dose-dependence of *E* could not be described by a sigmoidal curve but with a linear fit with a very slight slope (Figure 6). This reveals the exquisitely sensitive ability of the Laurdan FRET assay to discriminate between two conformational states (R and D) of the AChR. Furthermore, these results indicate that AChR conformational changes at the lipid−protein interface are coupled to the conformational changes of the whole AChR involved in the transition from the R to the D states.

DISCUSSION

Previous work from our laboratory using structurally different steroids demonstrated that these compounds act as NCI of the AChR (*8*, *33*−*34*, *37*). We considered several

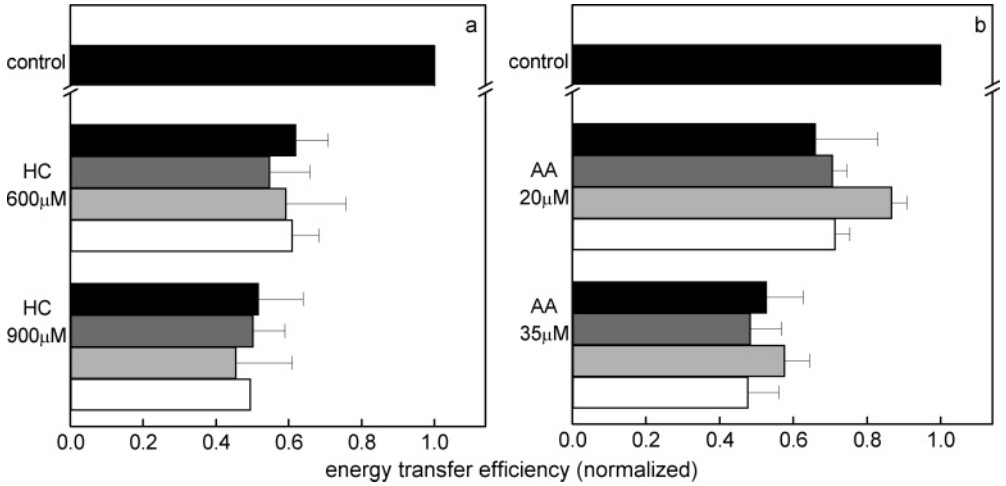

FIGURE 4: *E* for the AChR/Laurdan pair in *T. californica* rich-AChR membranes measured in samples submitted to controlled proteinase K proteolysis. Samples with 0% (black bars) 20−40% (gray bars), 40−60% (light gray bars) and 70% (white bars) hydrolysis in the presence of 0.6 μM Laurdan were exposed to different concentrations of (a) HC (600 and 900 μM); (b) AA (20 and 35 μM). The control bar corresponds to AChR-rich membranes in the presence of 0.6 μM Laurdan titrated only with solvent. Initial *E* values of 0.268 ± 0.019, 0.315 ± 0.024, 0.375 ± 0.021, 0.416 ± 0.022 were obtained in the absence of steroid or AA with samples having 0%, 20−40%, 40−60%, and 70% hydrolysis, respectively. Each bar corresponds to the average ± S.D. of at least five independent experiments. The differences between experimental conditions were not statistically significant.

hypotheses to explain the mechanism of action; the most plausible one appeared to be the allosteric induction of a nonconducting AChR conformation (*38*). In the present work, we studied the possible topology of sites for steroids and FFA, considered to act as NCI of the nicotinic receptor, in native AChR-rich membranes from *T. californica* using the efficiency of FRET between the intrinsic fluorescence of the protein and the fluorescence probe Laurdan.

In a previous work, we characterized the Trp−Laurdan pair as a good donor−acceptor FRET couple (*24*) with a Förster distance of 31 ± 1 Å, and a minimum donor−acceptor distance of 14 ± 1 Å (*24*), which corresponds roughly to the diameter of the first-shell AChR-associated ("boundary" or "annular") lipid (*3*). In that work, we described the occurrence of one type of sites for cholesterol and another type for phospholipids, both accessible to FFA (*16*), and subsequently showed that the access of FFA to both sites was independent of the FFA structure (i.e., number of carbon atoms and/or degree of saturation) (*17*).

The three steroids used in the present work were chosen because they exhibited the highest inhibitory potency within a large group of steroids tested by electrophysiology (*34*). Addition of increasing amounts of either steroid to the native membrane caused a diminution of *E* that is well fitted by a sigmoidal curve, implying a direct competition between Laurdan molecules and steroids (Figure 1). Further analysis of the data indicated the existence of more than one common site on the AChR oligomer (Figure 1 inset). Competition experiments indicated that different steroids share the same sites, as the addition of the second one caused no further variations in *E* (Figure 2). Additional experiments suggested that AA and HC also share a common binding site (Figure 3).

To delineate the topography of these sites, we treated native *T. californica* AChR-rich membranes with proteinase K, a soluble, nonspecific protease reported to hydrolyze the extracellular portions of the AChR (*12*, *30*, *35*). Samples with different degrees of digestion were studied by FRET. ΔE values were similar to those of control samples up to 70% digestion (Figure 4). Considering that the AChR is a membrane protein with a large extracellular domain, a TM domain, and a small intracellular domain, 70% digestion indicates almost complete extracellular hydrolysis (consistent with the postulated 70−75% AChR protein mass located outside the lipid bilayer (*12*, *30*, *35*)). The fact that sites for FFA and steroids could still be observed up to 70% digestion strongly suggests that these sites are located at the TM region and that the TM portion of the protein can still sustain its native conformation. This is further supported by cryoelectron microscopy data (*36*, *41*) indicating that the 20 TM segments in the whole AChR protein, arranged in three concentric rings, are stabilized mainly by protein−protein and/or protein−lipid interactions that occur locally (*2*, *42*). In addition, the present results discard the possibility that an allosteric mechanism involving extramembranous regions of the protein intervenes in the displacement of Laurdan from the lipid−AChR interface.

In living cells, a constant production of endogenous fatty acids occurs, and several roles have been attributed to this phenomenon. In some pathological situations, and in particular in neurons, the amount of endogenous FFA can increase dramatically, a phenomenon that has been interpreted as a neuroprotective mechanism (see refs *43*−*45*), although no consensus explanation has been found to account for the various actions of FFA on ion channel proteins (*46*, *47*). The experiments presented here, performed with membranes previously treated with PLA_2, show that endogenous FFA produced in situ are targeted to the lipid−protein interface, as visualized by the decrease in *E* values (Figure 5a). Laurdan molecules experience hindrance to occupy these sites and exogenous FFA compete with the FFA produced in situ for these sites (Figure 5b), lending further support to the occurrence of sites for FFA at the AChR TM region. This may constitute the common step in the mechanism of FFA modulation of AChR function in particular or other ion channels in general.

Two types of lipid sites have been described at the lipid−AChR (and membrane proteins, in general) interface: annular

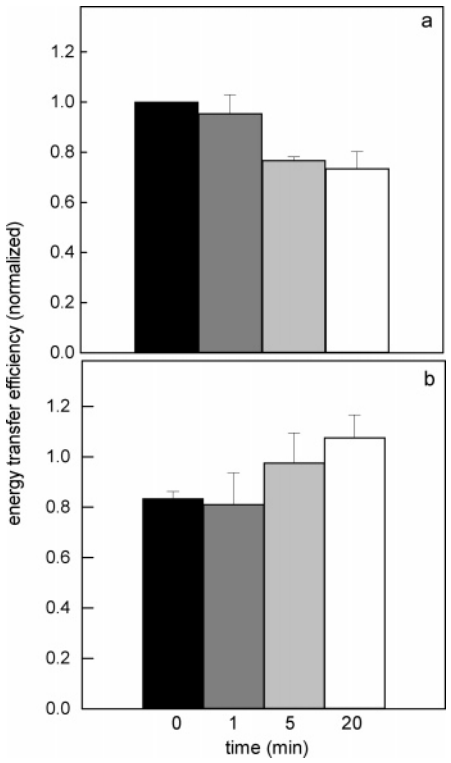

FIGURE 5: Effect of phospholipase A_2 treatment on normalized FRET efficiency between the intrinsic fluorescence of *T. californica* AChR membranes and Laurdan. (a) Changes in *E* as a function of time of phospholipid hydrolysis. The *E* value at time 0 of phospholipid hydrolysis was 0.277 ± 0.022. *E* values obtained at subsequent hydrolysis times were normalized with respect to the corresponding *E* value at time 0 (unity) (b) Changes in *E* in hydrolyzed membranes upon addition of 15 μM AA. The *E* values obtained in the presence of AA at different hydrolysis times were normalized with respect to the corresponding *E* value in the absence of AA. Each bar corresponds to the average $\pm$ S.D. of at least four independent experiments.

and nonannular sites. In general, nonannular lipid sites involve spaces between transmembrane α-helices and between subunits in multisubunit proteins (*18*). Nonannular lipids are considered essential for protein activity, interacting with higher specificity with the protein. The rate of exchange of nonannular lipid with bulk lipid has not yet been determined but could be relatively slow, and in any case much slower than that of annular lipid, as a result of the high specificity of the interaction between nonannular lipid and protein (*18*). On the other hand, lipid molecules at annular sites are purported to interact with proteins in a relatively less specific manner (*18*), and in the particular case of the AChR the rate of exchange of annular shell lipid and the bulk membrane lipid is fast (*3*). We surmise that at equilibrium the dwelling of FFA and steroids at nonannular sites is experimentally reflected by the steep initial phase of E. The monotonically linear decrease in E probably reflects mainly their diffusion and dilution with bulk lipids.

In the light of the current information on AChR structure down to 0.4 nm resolution stemming from cryoelectron microscopy data (*41*), the nonannular sites could correspond to crevices in between α-helical transmembrane peptides of the receptor protein. The results of a previous work from our laboratory (*16*) further support this view. We showed that E decreases in the presence of a typical annular lipid (phosphatidylcholine) induced only a slight, monotonically linear decrease of E, whereas a typical nonannular lipid (cholesterol) caused a steep initial decrease of E with subsequent saturation.

The results of the present work throw new light on lipid sites in AChR-rich membranes. We can now expand the concept of two distinct kinds of sites for cholesterol and phospholipids, respectively, both accessible to FFA (*16*), to include the notion that the two types of sites are also accessible to steroids. Agonist-induced ion flux is known to have an obligatory requirement for cholesterol (*19*−*21*), whereas steroids behave as noncompetitive antagonists of the AChR. Cholesterol and steroids display different affinities for the AChR (reviewed in ref 3). Furthermore, it has been demonstrated in model systems that steroids are lipid domain-disrupting compounds, whereas cholesterol and 25-hydroxycholesterol are lipid domain-promoting lipids (*39*). The main structural difference between cholesterol and steroids lies in the six-carbon side chain, and this may account for the difference in the ability of steroids to access the two types of sites whereas cholesterol only binds to one set of sites. These results are in agreement with published stopped-flow fluorescence measurements in which cholesterol and several analogues were postulated to be deeply located at the lipid−protein interface (*40*).

We addressed next the functionally relevant relationship between the occurrence of these sites and AChR functional states. For this purpose AChR preparations in the resting state were driven to the desensitized state and their response to the lipid ligands compared. Interestingly, the curves of *E* as a function of added exogenous ligand (FFA or steroid) showed a different pattern from that obtained when the AChR is in the resting state (Figure 6). In the D state, *E* decreases only slightly, in a linear and monotonical fashion, whereas in the R state, *E* drastically decreases in a sigmoidal form. Assuming that the steep initial decrease of *E* obtained in the resting AChR state corresponds to the displacement of Laurdan from nonannular sites, the absence of this slope in the desensitized state suggests that the nonannular sites are either no longer present or inaccessible. Thus, when the AChR is in the D state, the exogenous molecules only partition into the bulk lipid and annular lipid regions, displacing Laurdan from these regions, but this displacement does not result in substantial decreases in *E* because Laurdan molecules rapidly exchange between the two phases and their total number remains relatively constant. Thus, the notable difference in the ability of steroids and fatty acids to displace Laurdan from the AChR interface region between resting and desensitized conformations (Figure 6) can be most economically explained by the decreased efficacy of exogenous lipids in reaching the nonannular sites in the D state. AChR conformational transitions between the R state and the D state may thus entail a rearrangement of the AChR TM region involving the occlusion of sites at the lipid−protein interface or simply decreased lipid efficacy in accessing such sites.

The present study provides new insights into the possible modulatory mechanisms exerted by steroids and FFA on AChR function in particular and probably other membrane proteins in general. Changes in the membrane lipid composition (*19*−*20*, *22*, *48*) and/or the presence of exogenous hydrophobic molecules (*6*−*8*, *33*−*34*, *37*, *49*−*52*) affect AChR function. Individual amino acid mutations in the lipid-

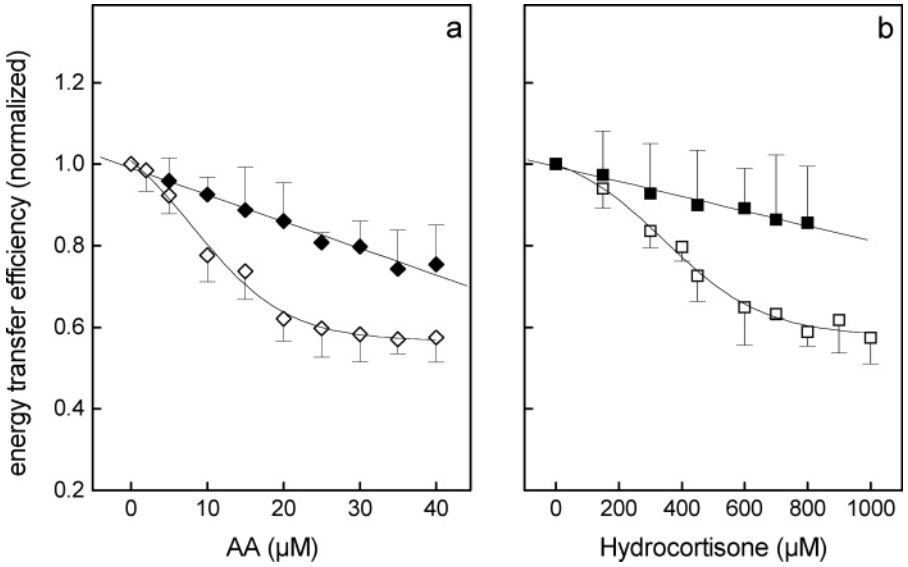

FIGURE 6: *E* of the FRET process between the intrinsic fluorescence of *T. californica* AChR membranes and Laurdan, measured in membranes with AChR in the resting state ("R", empty symbols) or desensitized state ("D", filled symbols). The latter was generated by incubation of the membrane with 1 mM Carb prior to the fluorescence measurements. The initial *E* values in the absence of exogenous lipids were 0.275 ± 0.018 and 0.295 ± 0.024 for the AChR in the R and D state, respectively. (a) Changes in *E* upon addition of increasing concentrations of AA (◆, ◇) or (b) HC (■, □). Each point corresponds to the average of at least eight independent measurements. See statistical analysis in the text.

facing M4 AChR TM domain also modulate AChR function (*53*−*58*), a clear indication that although far away from the agonist sites and from the ion channel, the M4 TM segment, the only member of the outermost TM ring (*2*), effectively influences AChR function. In this sense, M4 would behave as a sensor of the lipid−protein environment unleashing a signal to the M2 channel region, probably initiated by the induction of changes in the topology of the outer ring and ultimately causing a conformational change of the whole AChR (*42*). We would like to propose that FFA or steroids may induce, or contribute to this phenomenon, by perturbing nonannular sites. This mechanism would differ from the one exerted by the other group of NCI, the ion-channel blockers, postulated not to cause conformational changes in the AChR protein (*59*). Further studies will be necessary to dissect the sequence of steps linking the presence of highly hydrophobic compounds at the nonannular sites of the AChR with the epiphenomenological modulation of AChR function.

ACKNOWLEDGMENT

Thanks are due to Prof. Dr. M. Aveldaño and Dr. N. Furland for help in the lipid biochemical studies.

SUPPORTING INFORMATION AVAILABLE

TLC of lipid extracts isolated from *T. californica* AChR-rich membranes submitted to phospholipase A_2 (PLA_2) treatment to evaluate the endogenous production of FFA. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI061388Z