Physical State of Bulk and Protein-Associated Lipid in Nicotinic Acetylcholine Receptor-Rich Membrane Studied by Laurdan Generalized Polarization and Fluorescence Energy Transfer

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ABSTRACT The spectral properties of the fluorescent probe laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) were exploited to learn about the physical state of the lipids in the nicotinic acetylcholine receptor (AChR)-rich membrane and compare them with those in reconstituted liposomes prepared from lipids extracted from the native membrane and those formed with synthetic phosphatidylcholines. In all cases redshifts of 50 to 60 nm were observed as a function of temperature in the spectral emission maximum of laurdan embedded in these membranes. The so-called generalized polarization of laurdan exhibited high values (0.6 at 5°C) in AChR-rich membranes, diminishing by ~85% as temperature increased, but no phase transitions with a clear T_m were observed. A still unexploited property of laurdan, namely its ability to act as a fluorescence energy transfer acceptor from tryptophan emission, has been used to measure properties of the protein-vicinal lipid. Energy transfer from the protein in the AChR-rich membrane to laurdan molecules could be observed upon excitation at 290 nm. The efficiency of this process was ~55% for 1 μM laurdan. A minimum donor-acceptor distance r of 14 ± 1 Å could be calculated considering a distance $0 < H < 10$ Å for the separation of the planes containing donor and acceptor molecules, respectively. This value of r corresponds closely to the diameter of the first-shell protein-associated lipid. A value of ~1 was calculated for K_r, the apparent dissociation constant of laurdan, indicating no preferential affinity for the protein-associated probe, i.e., random distribution in the membrane. From the spectral characteristics of laurdan in the native AChR-rich membrane, differences in the structural and dynamic properties of water penetration in the protein-vicinal and bulk bilayer lipid regions can be deduced. We conclude that 1) the physical state of the bulk lipid in the native AChR-rich membrane is similar to that of the total lipids reconstituted in liposomes, exhibiting a decreasing polarity and an increased solvent dipolar relaxation at the hydrophilic/hydrophobic interface upon increasing the temperature; 2) the wavelength dependence of laurdan generalized polarization spectra indicates the presence of a single, ordered (from the point of view of molecular axis rotation)-liquid (from the point of view of lateral diffusion) lipid phase in the native AChR membrane; 3) laurdan molecules within energy transfer distance of the protein sense protein-associated lipid, which differs structurally and dynamically from the bulk bilayer lipid in terms of polarity and molecular motion and is associated with a lower degree of water penetration.

INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is an integral membrane protein, deeply embedded in the postsynaptic region of muscle, electrocyte, and nerve cells. There is an increasing body of evidence to support the notion that the function of this paradigm rapid ligand-gated receptor is influenced by its lipid microenvironment (see reviews in Barrantes, 1989, 1993a,b). Since the discovery of the immobilized lipid belt surrounding the AChR (Marsh and Barrantes, 1978), the concept of the "lipid annulus" or first-shell lipid belt region around this protein has become the focus of a variety of studies aimed at defining the nature of AChR-lipid interactions. The physical state of the membrane (for example, the so-called order parameter) has obviously received considerable attention (East et al., 1984). Fong and McNamee (1986), for instance, reported that reconstituted AChR is sensitive, within a narrow range of values, to the bulk fluidity of the membrane, as measured by ion flux experiments in vitro. More recently, Sunshine and McNamee (1994) varied the fluidity of the host lipid vesicle, as measured by steady-state fluorescence anisotropy of the probe diphenylhexatriene at a fixed temperature, and showed that AChR channel activity is maintained either in low- or high-fluidity environments. The authors suggested that lipid composition rather than fluidity determines the gating function of the channel.

To learn about the physical characteristics of the membrane in which the AChR is inserted and of the lipid belt region in particular, we have resorted here to the amphiphilic fluorescent probe laurdan (6-dodecanoyl-2-dimethylaminonaphthalene; Parasassi et al., 1986), whose synthesis followed the introduction of the parent compound Prodan by Weber and Farris (1979). Laurdan localizes itself at the hydrophilic-hydrophobic interface of the lipid bilayer (Chong, 1988, 1990), with its lauric acid moiety at the phospholipid acyl chain region and its naphthalene moiety

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at the level of the phospholipid glycerol backbone. Laurdan possesses an exquisite spectral 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 due to the effect of dipolar relaxation processes (Parasassi et al., 1990, 1991).

The spectroscopic properties of laurdan have been integrated into a parameter called generalized polarization (GP), introduced by Gratton and co-workers (Parasassi et al. 1990, 1991). Characteristic GP values in the pure gel and in the pure liquid-crystalline phospholipid phase have been determined. Thus, in a medium that can relax at a rate comparable to or faster than the fluorescence decay rate, such as the liquid-crystalline phase of phospholipids, excitation in the red part of the absorption band photoselects laurdan molecules that have already relaxed surroundings. The emission spectrum is dominated by the red band. By exciting the blue part of the absorption spectrum, laurdan molecules relax during their excited-state lifetime, emitting predominantly in the blue band with higher GP values. The additivity property of the GP can be used to quantify the relative proportion of coexisting phases in samples of unknown composition. GP measurements at more than one excitation or emission wavelength can help to determine whether domains of different composition and phase properties coexist in the membrane (Parasassi et al., 1993b). In pure liquid-crystalline systems moving the excitation wavelength from the blue to the red band of the excitation spectrum causes GP values to decrease. Phase coexistence in the membrane can be distinguished by the increase in the GP excitation values with increasing excitation wavelength, and a decrease in the GP emission values with increasing emission wavelength. This behavior apparently reflects fluorophore photoselection in different environments upon excitation at different wavelengths. In the present work we have taken advantage of these properties of laurdan to learn about the physical characteristics of the lipid milieu in the membrane where the AChR protein is embedded. We find differences between the AChR protein-associated lipid and the bulk lipid in the rest of the bilayer. Future work is aimed at defining the functional implications of these differences.

EXPERIMENTAL PROCEDURES

Materials

Torpedo marmorata specimens were obtained either from the Roscoff marine station in France or from the Mediterranean coast off Alicante, Spain. The latter were kindly provided by Dr. J. M. González-Ros. Fish were transported by ground in sealed plastic bags containing sea water and oxygen. On arrival they were killed by pithing, and the electric organs were dissected and stored at −70°C until further use. L-α-Dimyristoyl-phosphatidylcholine, HEPES, and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). Laurdan was purchased from Molecular Probes (Eugene, OR).

Methods

Preparation of AChR-rich membranes

Membrane fragments rich in AChR were prepared from the electric tissue of *T. marmorata* as previously described (Barrantes, 1982). Typically, specific activities on the order of 1.2–1.8 nmol α-bungarotoxin sites/mg protein were obtained.

Preparation of endogenous lipid extracts from AChR-rich membranes and their phospholipid analysis

Endogenous lipids were extracted from *T. marmorata* AChR-rich membranes by the method of Folch-Pi et al. (1957). Phospholipids were assayed as described by Rouser et al. (1970).

Preparation of liposomes

DMPC vesicles or liposomes prepared from the endogenous lipids present in the AChR-rich membrane were obtained by resuspending the mixed dried lipid extracts in 20 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 0.25 mM $MgCl_2$ to give a final concentration of 1 mg lipid/ml. Lipid dispersions were sonicated to clarity with a model W140 Sonifier cell disruptor. Sonication was carried out intermittently for 30–60-s periods with intervals of 1 min.

Fluorescence measurements

AChR-rich membranes and liposomes prepared from the endogenous lipids extracted from such membranes were resuspended in 20 mM HEPES buffer, 150 mM NaCl and 0.25 mM $MgCl_2$ (pH 7.4), and laurdan was added from a stock solution, resulting in a final probe concentration of 0.3 μM. Steady-state fluorescence measurements were carried out in the temperature range of 5 to 55°C using an SLM 4800 spectrofluorimeter (5 nm excitation and emission slits) and 10 × 10 mm quartz cuvettes. Emission spectra were corrected for wavelength-dependent distortions.

To investigate the occurrence of FRET between octyl-tryptophan and laurdan, small unilamellar vesicles (SUVs) of egg phosphatidylcholine (PC) containing octyl-Trp and different concentrations of laurdan were prepared by the ethanol injection method of Fung and Stryer (1978). The quantum yield of octyl-Trp in PC-SUV was 0.29 at 25°C, using as reference a quantum yield of 0.20 for tryptophan at pH 7.0 (see Fasman, 1976, and references therein). PC-SUV without octyl-Trp and varying concentrations of laurdan were used as controls and to correct for scattering effects. Because of the self-quenching of octyl-Trp it was technically impossible to obtain a complete FRET curve from the emission of laurdan at concentrations of octyl-Trp relative to phosphatidylcholine (on a molar basis) above 5%. On the other hand, at concentrations below 5% the light scattering contribution to the emission became significant, contributing up to 10% of the total signal. Furthermore, because of the fact that the quantum yield of laurdan emission is much higher than that of octyl-Trp, the calculation of FRET from the increase in laurdan emission was not undertaken beyond a laurdan concentration of 2% relative to the phosphatidylcholine concentration. The measurement of the quenching of octyl-Trp emission was finally performed using final concentrations of 5% octyl-Trp and 1% laurdan and quantitated in terms of arbitrary units obtained from the corresponding donor/acceptor emission spectral areas. Laurdan emission arising from excitation of this probe at 290 nm (less than 2% of the total signal even at 6% mol/mol laurdan/phosphatidylcholine ratios) and scattering components (less than 10%) were subtracted in each case.

Excitation GP (Parasassi et al., 1990, 1991) was calculated according to

$$\mathrm{exGP} = (I_{434} - I_{490})/(I_{434} + I_{490}), \quad (1)$$

where I_{434} and I_{490} are the emission intensity at the characteristic wavelength of the gel phase (434 nm) and the liquid-crystalline phase (490 nm), respectively. The exGP values were obtained from emission spectra at

different excitation wavelengths (320–410 nm) or at only one excitation wavelength where indicated. The emission GP was calculated according to the following formalism:

$$\text{emGP} = (I_{410} - I_{340})/(I_{410} + I_{340}), \quad (2)$$

where I_{410} and I_{340} are the excitation intensities at the wavelengths corresponding to the gel (410 nm) and the liquid-crystalline (340 nm) phases (Parasassi et al., 1993a). The emGP values were obtained from excitation spectra at different emission wavelengths (420–500 nm) or at only one emission wavelength where indicated.

The efficiency of the resonance energy transfer (E) in relation to all other deactivation processes of the excited donor depends on the sixth power of the distances between donor and acceptor. According to Förster's theory (Förster, 1948) E is given by

$$E = R_o^6 / (R_o^6 + r^6), \quad (3)$$

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%. R_o can be calculated from the equation

$$R_o = 9.79 \times 10^3 (J\eta^{-4}\kappa^2\Phi_D)^{1/6} \text{ (Å)}, \quad (4)$$

where η is the refractive index of the medium, κ^2 is the orientation factor between the transition moments of the donor and acceptor, Φ_D is the fluorescence quantum yield of the energy donor in the absence of acceptor, and J is the spectral overlap integral. The latter is given by

$$J = \int F(\nu)\epsilon(\nu)\, \nu^4\, d\nu \, (\text{M}^{-1}\,\text{cm}^3), \quad (5)$$

where $F(\nu)$ is the fluorescence spectrum of the donor normalized to unity, and $\epsilon(\nu)$ is the absorption spectrum of the acceptor. Alternatively, the excitation spectrum of laurdan in AChR-rich membrane vesicles was used, given the difficulties in obtaining the absorption spectrum in these samples, as previously encountered with other probes (Valenzuela et al., 1994). The fluorescence excitation spectrum of the membrane-associated fluorophore has been taken as representative of the corresponding absorption spectrum (Valenzuela et al., 1994). The J value was calculated from the excitation spectrum of laurdan in AChR-rich membranes and the intrinsic emission spectrum of the AChR-rich membrane from *T. marmorata*, which show a high degree of overlap. A value of $J = 2.3 \times 10^{-14}\ \text{M}^{-1}\ \text{cm}^3$ was obtained for this pair. A value of $J = 1.59 \times 10^{-14}\ \text{M}^{-1}\ \text{cm}^3$ was obtained for the octyl-Trp/laurdan pair, showing an excellent correlation between the two sets of data. A value of 1.33 was used for the refractive index of the medium, and 2/3 for κ^2, as in Valenzuela et al. (1994) and Gutiérrez-Merino et al. (1995). The latter value corresponds to a rapid random reorientation of the donor and acceptor transition electronic moments. On strict theoretical grounds, the value of $\kappa^2 = 2/3$ only applies to a 3D isotropic distribution of both donors and acceptors, and with some limitations to a 3D distribution of donors and a plane (2D distribution) of acceptors. The effect of choosing a slightly different value for κ^2 or a range for the parameter as an appropriate alternative would not substantially affect the calculations, but would have complicated the statistical analysis. Furthermore, the experimental data on FRET with the octyl-Trp/laurdan pair incorporated into small unilamellar vesicles were fitted extremely well using a value of $\kappa^2 = 2/3$.

Measurements of the extent of quenching of donor fluorescence by Förster energy transfer in the absence and presence of increasing concentrations of laurdan were carried out using an SLM 4800 spectrofluorimeter. Emission spectra were corrected for wavelength-dependent distortions. Inner-filter effects were small and were corrected as described by Lakowicz (1983) and Homan and Eisenberg (1985). Correction of inner-filter effects also implicitly takes into account the turbidity of AChR membranes. Fluorescence measurements were carried out in such a way that changes in the turbidity of the samples were kept below 0.05 O.D. units throughout the experiment (note that laurdan was added at a low molar ratio with respect to lipid (~1%), and on average only about 50 μg AChR membrane protein and 35 nmol phospholipids were used in 1.5-ml cuvettes). To assess whether lipid peroxidation occurred in AChR-rich membranes under the experimental conditions used, laurdan GP was measured under direct (360 nm) or FRET excitation (290 nm) after extensively bubbling the AChR-rich membranes with O_2 or N_2. The differences between the GP values under the two excitation conditions were maintained throughout in samples that were measured under equilibration with either gas, and no time-dependent changes were observed in the course of these experiments (data not shown).

Analysis of Förster energy transfer data

The analysis of the fluorescence energy transfer data, obtained by assessing the degree of donor (protein intrinsic fluorescence) quenching (see above), was carried out as indicated in Gutiérrez-Merino et al. (1994, 1995). The method was calibrated using experimental data for the fluorescein isothiocyanate–phosphatidylethanolamine/rhodamine-phosphatidylcholine and fluorescein isothiocyanate–phosphatidylethanolamine/XRITC-phosphatidylethanolamine donor/emission pairs and simulated using the data of Fung and Stryer (1978) for various other pairs. Briefly, the overall rate of the energy transfer of the ensemble (k_T) can be computed as the sum of the energy transfer occurring between individual donor-acceptor pairs in the two-dimensional membrane system (the lipid bilayer), i.e., $k_T = \Sigma k_i$, where the rate of transfer for pair i separated by a distance r_i is k_i, provided that the translational diffusion rate of the donor and acceptor is much slower than the lifetime of the excited state of the donor. We have experimentally measured AChR translational diffusion, having obtained a value of D of $1–3 \times 10^{-8}\ \text{cm}^2\ \text{s}^{-1}$ between 14°C and 37°C (Criado et al., 1982); the condition is thus met. Because of space and time averaging, the assumption of random orientation between donor and acceptor appears to be fully justified under the present experimental conditions (see, e.g., Gutiérrez-Merino et al., 1987). The overall efficiency of the fluorescence energy transfer, $\langle E \rangle$, can be written as

$$\langle E \rangle = k_T/k_o + k_T, \quad (6)$$

where k_o is the value of the average rate constant of energy transfer for a donor-acceptor pair separated by the characteristic distance R_o, i.e., the distance at which the efficiency of energy transfer is equal to 50%.

The analytical approach developed by Gutiérrez-Merino et al. (1987, 1994, 1995) was used to calculate the average rate of Förster energy transfer as a function of the position of donor in the membrane protein with respect to the plane of acceptors. This approach envisages both donor and acceptor molecules in the membrane as a continuum of discs of different sizes, to compute all the possible donor-acceptor pairs contributing to FRET at a given distance r_i. For this purpose the Trp residues were assumed to lie in a ring within the perimeter of the transmembrane portion of the AChR that has an outer radius of 32.5 Å (the diameter of this portion of the receptor protein is 65–70 Å; cf. Unwin, 1993). Trp residues in the AChR can be expected to undergo homotransference of energy between themselves (hence their low anisotropy and average low quantum yield) and be organized in a network in the three-dimensional structure of the AChR. The height of the plane of AChR tryptophan residues was set with respect to the plane of acceptors by using the parameter H, the distance between donor-acceptor planes normal to the membrane surface, which was allowed to vary between 0 and 50 Å in view of the long-axis dimensions of the AChR molecule and the width of the lipid bilayer (Unwin, 1993). The AChR cylinder was in turn assumed to be distributed in a regular triangular lattice in the host bilayer, also based on the maximum packing habits of two-dimensional ordered arrays of AChR particles from which the structural information was derived (Unwin, 1993). In this view, the AChR molecule is surrounded by a belt of lipid molecules of ~10 Å diameter each. The surface area not accessible to acceptor molecules owing to the perturbation introduced by neighboring AChR molecules has been taken into consideration following the treatment of Gutiérrez-Merino et al. (1987). The plane of acceptors was simulated assuming an average area per lipid molecule of 0.75 nm^2 (Rand, 1981). Laurdan molecules,

whose concentration with respect to endogenous lipids was always kept very low (see text), were assumed to have a similar cross section (Parasassi et al., 1986) and to be located at the water/polar headgroup interface (Chong, 1988, 1990). The distance r between the donor Trp ring in the AChR molecule and acceptor laurdan molecules in vicinal lipids around the AChR protein has been taken as the distance of closest approach between donor-acceptor pairs. In the simulations this parameter r was allowed to vary between 10 Å, i.e., approximately the sum of the van der Waal radii of tryptophan and laurdan, and 35 Å, the maximum radius of the AChR molecule. Random and nonrandom distribution (i.e., preferential binding) of laurdan in the annular lipid was introduced in the calculation as follows. The probability (α) of occupancy of sites at the lipid belt region by the fluorescent lipid laurdan (L_1) relative to that of unlabeled lipids has been calculated from its dissociation constant relative to the average dissociation constant of unlabeled lipids in the bilayer (L_2). Thus, using the equilibria

$$P + L_1 \approx PL_1, \quad \text{with } K_1 \text{ as dissociation constant} \qquad (7)$$

$$P + L_2 \approx PL_2, \quad \text{with } K_2 \text{ as dissociation constant} \qquad (8)$$

and expressing the concentrations of L_1 and L_2 as molar fractions, x_1 and x_2 respectively, it follows that

$$\alpha = (x_1/K_1)/(x_2/K_2) = (x_1/x_2) \cdot K_r^{-1}, \qquad (9)$$

where $x_1 + x_2 = 1$ and where K_r is the apparent dissociation constant of laurdan for the lipid belt region.

The values of energy transfer efficiency for different acceptor surface densities were calculated as a function of r, H, and K_r by parametric fitting of the experimental data to theoretical predictions of FRET by weighting the probability of occurrence of donor/acceptor pairs at the lipid belt region relative to that of a random distribution. K_r provided a measure of laurdan's distribution in the AChR membrane. For the case of $K_r = 1$, it follows that the probability of occupancy of each annular site by laurdan is $\alpha = x_1/x_2$, as expected for a random distribution of probe molecules in the bilayer. In this case the probability of occupancy of sites by laurdan at the lipid belt region is the same as the probability of laurdan molecules being at any other location in the lipid bilayer, such that its weighting factor is simply the molar ratio of the two possible locations. $K_r < 1$ implies preferential location of laurdan in the lipid belt region, and $K_r > 1$, laurdan's exclusion from this region. We drew theoretical curves for different R_o, r, and H values. First, by comparing the experimental data with different sets of theoretical curves, a value of r was deduced. Different theoretical curves were also furnished for sets of K_r values varying from 0.01 to 100 by using a fixed R_o. By comparing these curves with the experimental data, a value of K_r was obtained. Deviation from a random distribution of laurdan in the plane of the lipid bilayer can thus be simulated in terms of different affinities of labeled and unlabeled lipids for the AChR-lipid interface.

RESULTS AND DISCUSSION

Laurdan fluorescence properties in the bulk lipid of the AChR-rich membrane

Emission spectra of laurdan incorporated into the AChR-rich membrane from *T. marmorata* (or from *D. tschudii*; not shown) upon direct excitation at 360 nm are shown in Fig. 1 *a*. The emission maximum was centered at 434 nm at low temperatures and redshifted as the temperature increased. Laurdan emission spectra were further characterized by the appearance of an additional band centered at a wavelength of about 490 nm at higher temperatures (Fig. 1 *a*). This emission band has been observed with phospholipids in the liquid-crystalline phase, in which the rate of dipolar relaxation is high (Parasassi et al., 1991). The behavior of laurdan fluorescence incorporated into liposomes prepared from lipids extracted from *T. marmorata*, also excited at 360 nm (Fig. 1 *b*), showed the same wavelength and temperature dependence as that of the native AChR-rich membrane (the same applies to *D. tschudii*; not shown). These results indicate that the polarity and the dynamics of the bulk lipid are almost the same in the native membrane and in reconstituted liposomes devoid of protein but with a high endogenous cholesterol content (see Barrantes, 1989). By comparison with results obtained with model systems (Parasassi et al., 1991, 1993a,b), the behavior of laurdan emission as a function of temperature indicates higher mobility of the membrane constituents at the hydrophilic-hydrophobic interface (the glycerol backbone) of the bulk lipid, which can be attributed, in turn, to a temperature-dependent increase in the dipolar relaxation in that region, sensed by the probe as the temperature is augmented.

The so-called GP of laurdan is defined by the following algorithm (Parasassi et al., 1986):

$$GP = (I_B - I_R)/(I_B + I_R), \qquad (10)$$

where I_B and I_R are the fluorescence intensities at the blue and red edges of the emission spectrum, respectively. Fig. 2 shows the temperature dependence (5–

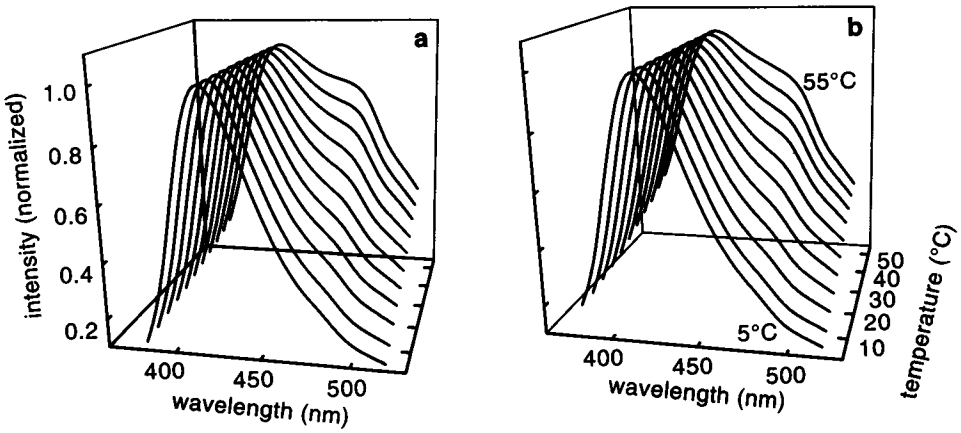

FIGURE 1 Normalized emission spectra of laurdan in the AChR-rich membrane from *T. marmorata* (*a*) and in liposomes of lipid extracted therefrom (*b*) as a function of temperature (5–55°C). Excitation wavelength: 360 nm. Excitation and emission slits: 5 nm. The laurdan/membrane lipid ratio was 1:150.

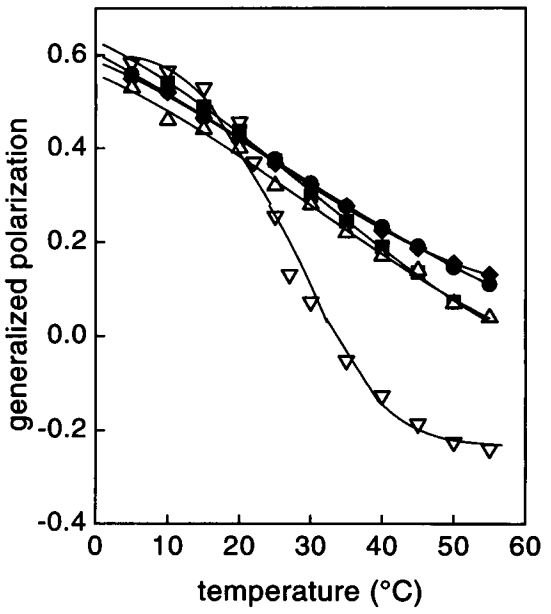

FIGURE 2 Excitation GP of laurdan in the AChR-rich membrane from *T. marmorata* (●) and from *D. tschudii* (■) electric organ, and in liposomes prepared from lipids extracted from AChR membranes from *T. marmorata* (◆) and from *D. tschudii* (△), and model lipid vesicles (DMPC, ▽) as a function of temperature. GP values were calculated from spectra like those shown in Fig. 1, using the emission wavelengths at 434 and 490 nm. The laurdan/membrane lipid ratios were 1:400, 1:150, and 1:400, respectively. In this and the remaining figures each point corresponds to the average ± SD of at least three determinations.

55°C) of laurdan exGP in the AChR-rich membrane and in liposomes prepared from their lipid extracts. In the gel phase exGP values of ≥0.5 are expected (Parasassi et al., 1990, 1991). Considering that the temperature of the *Torpedinidae* natural habitat is usually 5–15°C, it is reasonable that the exGP values of laurdan in the native AChR-rich membrane and liposomes prepared from their extracted lipids are also maintained in vitro within relatively high values (0.5–0.6) at these relatively low temperatures. For comparative purposes the exGP values for pure DMPC liposomes are also shown; at low temperatures DMPC liposomes are in the gel phase, whereas at room temperature and above, they are in the liquid-crystalline phase, undergoing a phase transition centered at 23°C (Fig. 2). Both in the AChR-rich membrane and in the liposomes prepared from their lipid extracts the exGP values were qualitatively similar to those of DMPC vesicles up to about 15°C. At variance with DMPC, they exhibited a steady, progressive diminution of exGP with increasing temperature, indicating that the membranes did not undergo distinct phase changes but a gradual modification of order and mobility parameters and environmental polarity as a function of temperature. Phase changes would have been manifested by a critical diminution of the GP values within a narrow range of temperatures, as is the case with DMPC.

Tryptophan and laurdan constitute a donor-acceptor pair for fluorescence energy transfer

Fig. 3 *a* shows the emission spectra of 5% octyl-Trp incorporated into phosphatidylcholine (PC) SUVs, and the same upon addition of laurdan at a molar ratio of 1% with respect to the total phospholipid. The magnitude of the quenching of octyl-Trp emission (69.7 a.u.) correlates with the concomitant increment in laurdan emission after subtraction of the laurdan emission arising from excitation of this probe at 290 nm (74.5 a.u.).

We also extracted quantitative information on the relative topography of this donor-acceptor pair, following the theoretical approach developed by Gutiérrez-Merino (1981) and by Gutiérrez-Merino et al. (1987, 1994). Assuming a ran-

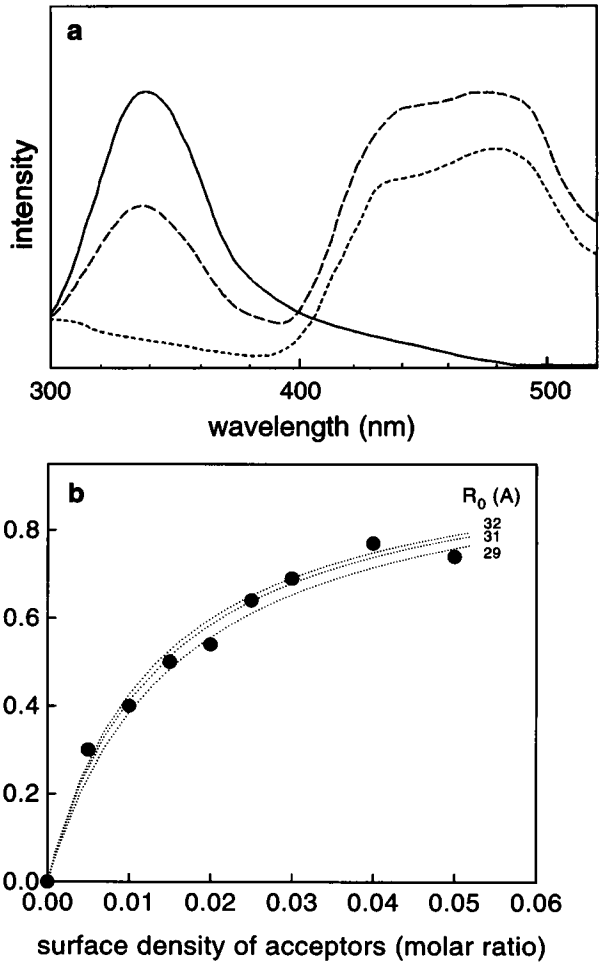

FIGURE 3 (*a*) Emission spectra of 5% octyl-Trp incorporated into phosphatidylcholine (PC) small unilamellar liposomes (——), the same upon addition of laurdan at a molar ratio of 1% with respect to the total phospholipid (– – –), and liposomes with 1% laurdan but no octyl-Trp (·····). (*b*) Efficiency of resonance energy transfer and calculated distances between the two members of the donor-acceptor pair (octyl-Trp and laurdan) in PC-SUV as a function of the surface density of energy transfer acceptors. The theoretical curves correspond to different R_0 values, calculated according to the method of Gutiérrez-Merino (1981). The final PC concentration was 40 μg/ml. Excitation wavelength was 280 nm; intrinsic protein emission was measured at 340 nm. The temperature was 25°C.

dom distribution of donors and acceptors within the membrane, R should represent an average donor-acceptor distance. E can also be defined as

$$E = 1 - (\Phi/\Phi_D) \approx 1 - (I/I_D), \qquad (11)$$

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. Fig. 3 *b* quantitatively depicts the occurrence of fluorescence energy transfer between octyl-Trp and laurdan. An R_o value of 31 ± 1 Å could be calculated for this donor/acceptor pair (see Experimental Procedures).

Laurdan fluorescence properties at the AChR-lipid interface

The emission spectrum of the AChR-rich membrane from *T. marmorata* and the excitation spectrum of laurdan incorporated into these membranes are shown in Fig. 4. The intrinsic protein emission spectrum of the AChR-rich membrane protein, centered at 335–340 nm (Barrantes, 1978), is dominated by tryptophan emission. The excitation spectrum of laurdan incorporated into the native AChR-rich membrane exhibited a maximum at 365 nm. Spectral overlap between the emission spectrum of the intrinsic protein fluorescence (tryptophan residues), acting as the donor, and the excitation spectrum of laurdan, as the acceptor, is an essential requirement for establishing fluorescence resonance energy transfer (FRET) conditions. Excitation under FRET conditions using the tryptophan residues of the AChR-rich membrane as donors was used to obtain information on the properties of the lipids in the immediate vicinity of the AChR in comparison to the average, bulk lipid in the rest of the bilayer. From the spectral overlap integral for the AChR membrane-laurdan pair (the corrected emission spectra of the AChR-rich membrane intrinsic protein and the absorption spectrum of laurdan; cf. Fig. 4 *a*) we calculated R_o, the Förster critical distance, to be 29 Å. The small difference between this value and that found for the octyl-Trp/laurdan pair could be attributed to small differences in the laurdan absorption spectrum in the native membrane and the SUV, respectively, due in turn to different properties at the polar head region-water interface (e.g., curvature) of the two systems. The concomitant decrease in donor emission and increase in acceptor emission upon increasing the concentration of laurdan in the AChR membrane, attributable to the occurrence of fluorescence energy transfer, is shown in Fig. 4 *b*.

Information on the relative topography of donor and acceptor molecules could also be obtained for the intrinsic protein-laurdan pair following the theoretical approach previously applied to the model octyl-Trp/laurdan pair. We modeled the AChR as a cylinder of about 80 Å diameter with donor Trp residues lying in a ring within the perimeter of its transmembrane portion. This region of the protein was assumed to have a radius of 32.5 Å (cf. Unwin, 1993). The height of the plane of AChR tryptophan residues was set with respect to the plane of acceptors by using the parameter H, the distance between donor-acceptor planes normal to the membrane surface, which was allowed to vary between 0 and 50 Å in view of the long-axis dimensions of the AChR molecule and the width of the lipid bilayer (Fig. 5). As expected for a constant molar ratio of the donor AChR protein in the membrane, E varied as a function of laurdan acceptor surface density. The efficiency of FRET in the *Torpedo* AChR-rich membrane reached values of about 50% for molar acceptor/total lipid ratios of 0.03 or higher (Fig. 6). Theoretical curves for different values of the minimum donor-acceptor distance, r, and different separations between the planes of donor and acceptor, respectively, H, were generated (see Experimental Procedures). Fig. 6, *a* and *b*, shows the sets of

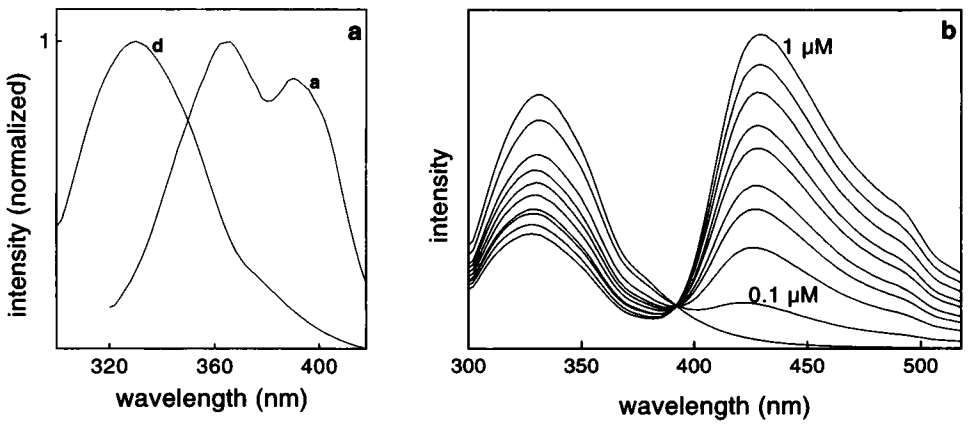

FIGURE 4 (*a*) Spectral overlap between the emission spectrum of the AChR membrane intrinsic protein fluorescence (*d*, donor) excited at 290 nm and the excitation spectrum of laurdan (*a*, acceptor) at an emission wavelength of 440 nm, the main emission band of laurdan. Spectra were obtained at 20°C. A value of $J = 5.3 \times 10^{15}$ cm^3 M^{-1} was obtained (see Experimental Procedures). (*b*) Emission spectra of laurdan in *T. marmorata* AChR-rich membrane under FRET conditions (constant molar ratio of the donor AChR protein in the membrane) as a function of acceptor surface density. The intrinsic protein fluorescence displays a maximum at 330 nm, and laurdan emission shows two peaks between 400 and 500 nm. Laurdan concentration was varied between 0.1 and 1 μM. The temperature was 20°C.

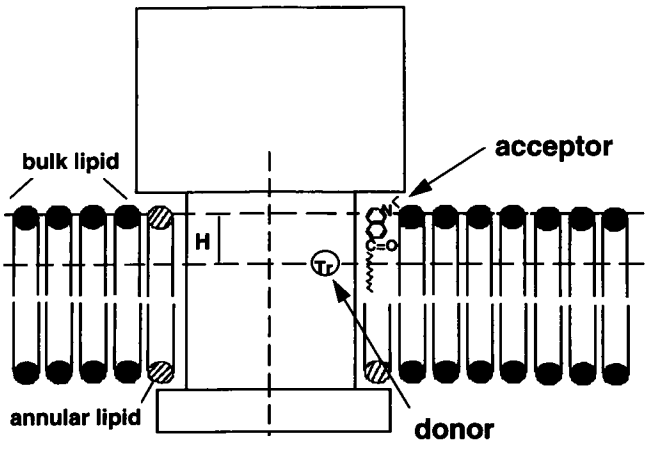

FIGURE 5 Topographical relationship between the membrane-bound AChR, surrounding lipid molecules, and laurdan in cross-sectional representation. The low anisotropy of the AChR intrinsic fluorescence indicates that it is highly likely that Trp residues are arranged in a network, with extensive FRET occurring among individual members of such an array. Only one Trp residue (Tr) is represented in the AChR protein for the sake of clarity. This Trp acts as the donor to a laurdan molecule acceptor in the protein-vicinal lipid annulus (shaded circles indicate the polar head region of protein-vicinal phospholipid in the AChR lipid belt region). The orientation of the acceptor molecule and its depth in the membrane bilayer follow the data of Chong (1988, 1990) and Parasassi et al. (1991). H is the distance between the plane of the donor and that of the acceptor; r is the distance of closest approximation between the donor and acceptor molecules.

curves for $H = 0$ Å and 10 Å, respectively, together with the experimental data. An average value of $r = 14 \pm 1$ Å was chosen for H between 0 and 10 Å. This value matches the thickness of a single layer of phospholipid molecules of about 0.75 nm^2 surface area (Rand, 1981) and is in agreement with the presumptive location of laurdan in the bilayer (Chong, 1988, 1990; Parasassi et al., 1991) and with the average distance of 10 Å for the depth of AChR tryptophan residue(s) from the center of the bilayer calculated by Chattopadhyay and McNamee (1991).

Similarly, by comparing the experimental values with theoretical curves with different values of K_r between 0.01 and 100 (Fig. 7), a K_r value of $\cong 1$ was obtained, indicating that the distribution of laurdan in the lipid bilayer was random. This result is in agreement with the values of energy transfer efficiency; had laurdan had affinity for the lipid annulus, these values would be higher than 50% at a low laurdan:AChR ratio. Our results are also in agreement with the more general observation that laurdan fluorescence is independent of the type of polar headgroup in the membrane phospholipids (Parasassi et al., 1991).

Having established the occurrence of energy transfer between protein and laurdan in the AChR-rich membrane by steady-state FRET (Fig. 4), we studied next the temperature sensitivity of laurdan GP under direct excitation and FRET conditions (Fig. 8). Considering that the efficiency of the energy transfer process, E, depends on the sixth power of the donor-acceptor distance, and the experimentally determined R_o of 29 Å, laurdan molecules excited under FRET conditions are a fraction of the total population localized within a reduced volume around the AChR protein, at a maximum radius slightly larger than the magnitude of R_o. If direct excitation (360 nm) of laurdan reflects the bulk lipid physical state, and excitation of laurdan under FRET conditions (290 nm) is used as a probe of the lipid-AChR interface region, from comparison of the two sets of GP values the larger values found for the set obtained under FRET conditions (Fig. 8) indicate that the laurdan molecules within energy transfer distance (i.e., at the AChR-lipid

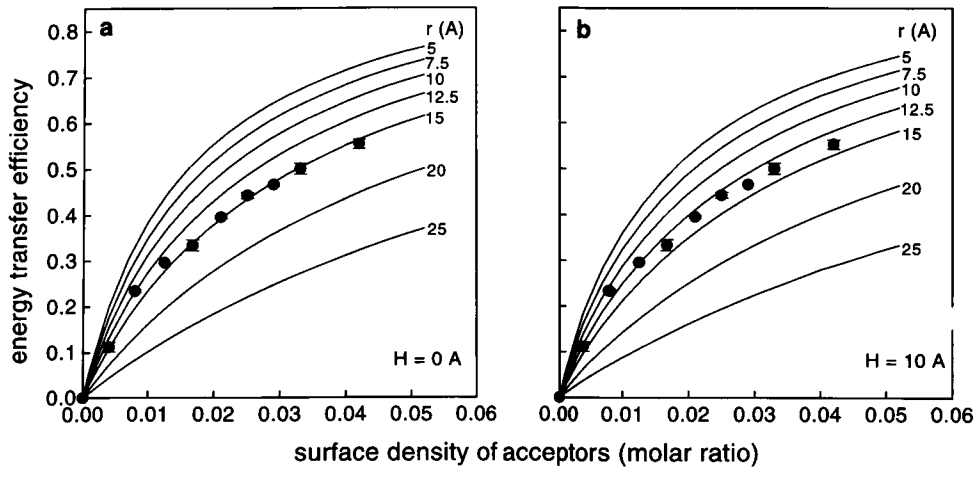

FIGURE 6 Efficiency of resonance energy transfer and calculated distances between donor-acceptor pairs (protein intrinsic fluorescence-laurdan) in the native AChR-rich membrane as a function of the surface density of energy transfer acceptors. A constant concentration of the fluorescent donor (the AChR protein) was titrated with increasing concentrations of the fluorescent acceptor. The curves (continuous lines) correspond to the theoretical fits to the donor-acceptor minimal distances, calculated according to the method of Gutiérrez-Merino (1981), and the symbols (●) correspond to the experimental values: (*a*) theoretical curves of different r, with $H = 0$ Å and $R_o = 29$ Å, and (*b*) theoretical curves for different r, with $H = 10$ Å and $R_o = 29$ Å (see Fig. 5). The concentration of protein in the AChR-rich membrane was kept at about 35 μg/ml; phospholipid concentration was 24 μM. Laurdan concentration was varied between 0.1 and 1 μM. The temperature was 20°C.

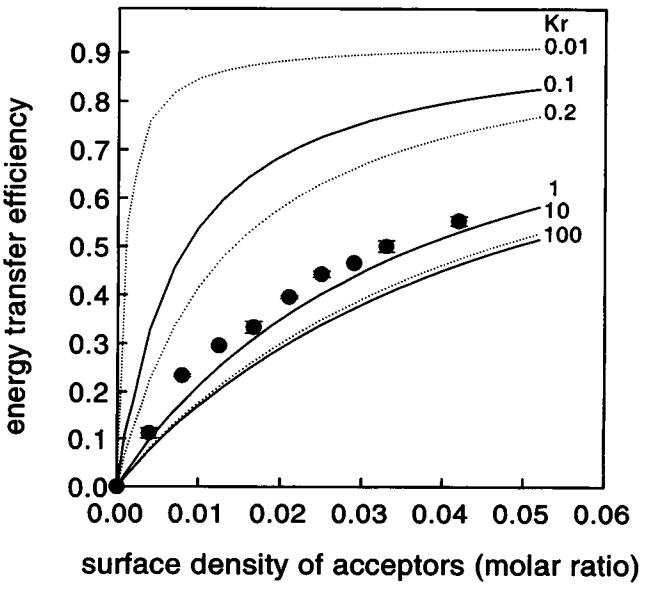

FIGURE 7 Efficiency of fluorescence energy transfer as a function of the surface density of acceptors (laurdan molecules) for different values of K_r, the apparent dissociation constant of laurdan for the lipid belt region surrounding the AChR protein in the native membrane. Theoretical curves (*continuous lines*), based on the treatment of Gutiérrez-Merino et al. (1987), were calculated for the different values of K_r indicated in the graph. The other parameters (see Fig. 5) were kept as follows: $H = 0$, $r = 15$ Å and $R_o = 29$ Å (see also Fig. 6). The experimental values (●) are closely approximated by a theoretical curve with K_r close to unity. The protein concentration in the AChR-rich membrane was kept at about 35 μg/ml; phospholipid concentration was 24 μM. Laurdan concentration was varied between 0.1 and 1 μM. The temperature was 20°C.

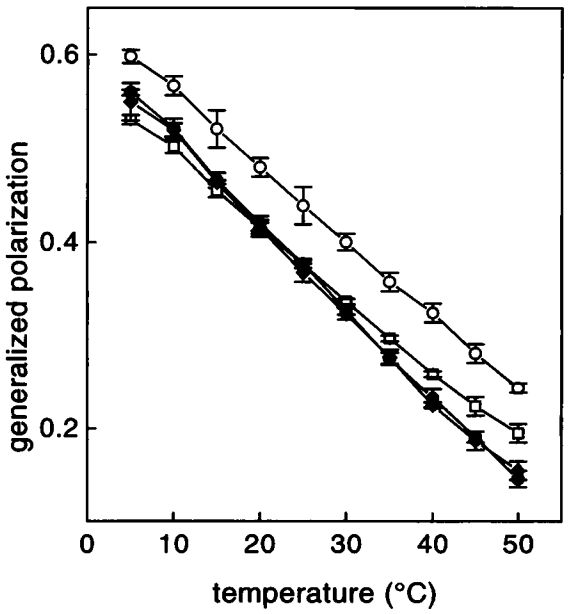

FIGURE 8 Comparison of laurdan exGP using direct excitation of the probe (●, 360 nm) or FRET from the protein in the *T. marmorata* AChR membrane (○, 290 nm) and of liposomes prepared from their extracted lipids (□, 290 nm; ♦, 360 nm). GP values were calculated for wavelengths of 434 and 490 nm. The laurdan/membrane lipid ratio was 1:200.

interface) lie in a relatively more rigid environment than those in the bulk lipid.

To ascertain the contribution of the protein to this phenomenon, GP values were also obtained as a function of temperature in liposomes prepared from lipid extracted from the *T. marmorata* AChR-rich membrane and compared with those of the native, parental membrane (Fig. 8). The general trend of the temperature profile of laurdan in the AChR membrane and liposomes prepared from their lipid extracts were essentially similar, overlapping up to a temperature of 30°C (Fig. 8). When the *T. marmorata* AChR-rich membrane was excited at 360 nm and liposomes prepared from the extracted endogenous lipids were excited at 290 and 360 nm, the GP values were very similar in the temperature range from 5°C to 50°C. But when laurdan was excited under FRET conditions in *T. marmorata* AChR-rich membranes, the GP values were significantly higher than those obtained by direct excitation at 360 nm, especially in the high temperature range (7% higher at 5°C and 67% higher at 50°C). This supports the assumption that the laurdan molecules within FRET distance, and by inference at the lipid-AChR interface region, lie in a relatively more rigid environment than the rest of the membrane, i.e., their mobility is lower than that of the bulk lipid, thus allowing for a lesser degree of water penetration.

Refinement of the data on the environment of laurdan molecules in the AChR-rich membrane

Parasassi et al. (1993b) suggested that further information about the environment of the reporter laurdan molecules can be obtained from the wavelength dependence of GP spectra. A wavelength independent GP spectrum is characteristic of the gel phase, whereas a liquid-crystalline phase GP typically exhibits wavelength dependence, due to the dipolar relaxation process, with decreasing values for the excitation GP spectrum and increasing values for the emission GP spectrum. When the two phases coexist the GP spectra show the opposite behavior, because of the photoselection process. Furthermore, when two phases coexist, high cholesterol concentrations (30% or more) reverse the above trend of the GP spectral dependence, such that there is no more photoselection and a spectral behavior similar to that of the liquid-crystalline phase (but with higher GP values) is apparent, suggesting the presence of a single phase in which a fast dipolar relaxation process occurs (ordered-liquid phase) (Parasassi et al., 1990). This phase shares properties typical of the liquid phase (lateral diffusion) and of the solid phase (ordered acyl chains) resulting from the "averaging" effect of cholesterol. These effects have been experimentally induced by the addition of exogenous cholesterol to liver microsomes (Castuma et al., 1991, 1993).

We studied GP in native *T. marmorata* AChR membranes and in liposomes prepared from their lipid extracts as a function of excitation (320–410 nm) and emission (420–500 nm) wavelengths at 20°C (Fig. 9). Excitation and emission GP values were calculated according to Eqs. 1 and 2

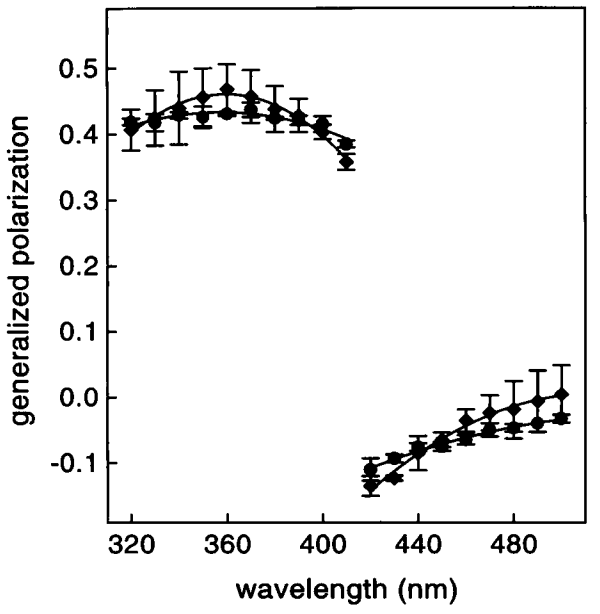

FIGURE 9 GP of laurdan in the AChR-rich membrane from *T. marmorata* (●) and in liposomes prepared from lipids extracted therefrom (◆) as a function of excitation (320 to 410 nm) and emission (420 to 500 nm) wavelengths, at 20°C. Values of emission at 434 and 490 nm were used for recording the excitation spectra of membrane and liposomes, respectively, and maxima at 340 and 410 nm were measured for the emission spectra, respectively. The laurdan/membrane lipid ratios were 1:100 and 1:200 for native membranes and liposomes, respectively.

above. The spectra exhibited decreasing values of the excitation GP and increasing values of the emission GP with increasing wavelengths (Fig. 9). This behavior indicates that dipolar relaxation occurs and that there is no selective excitation of subpopulations of laurdan molecules, thus suggesting that the environment of laurdan molecules in the native AChR-rich membrane displays the characteristics of the ordered-liquid phase described above. The pattern is also observed in cholesterol-rich membranes, as expected when taking into account the high cholesterol content of the AChR-rich membrane (see Barrantes, 1989). The steeper variations exhibited by both excitation and emission GP spectra of the liposomes prepared from the extracted lipids could indicate a higher heterogeneity of lipid domains in the absence of the protein. Furthermore, we interpret the higher absolute values of the pure lipid spectra to indicate that in the absence of the protein, cholesterol dominates the photophysical behavior of laurdan, decreasing both the polarity and the molecular motion of its environment (Parasassi et al., 1990, 1994). Castuma et al. (1991) have shown that cholesterol enrichment diminished the mobility and increased the order of bulk bilayer lipids, whereas it increased the mobility and decreased the order of annular, protein-associated lipid.

Fong and McNamee (1986) indicated a correlation between the ion flux properties of the reconstituted AChR and the membrane order parameter measured by electron spin resonance techniques. However, in more recent work from McNamee's laboratory (Sunshine and McNamee, 1994), no consistent correlation between the ion flux response in reconstituted AChR and the steady-state fluorescence anisotropy of the probe diphenylhexatriene, another technique to measure membrane order, could be found. To explain this discrepancy, Sunshine and McNamee (1994) suscribed to our concept of an AChR lipid belt region and elaborated a modified "optimal fluidity hypothesis": the possibility that it was the fluidity of the lipids directly surrounding the AChR, i.e., the AChR lipid annulus (Marsh and Barrantes, 1978), that could be necessary for optimal receptor function. Narayanaswami and McNamee (1993) interpreted their data as reflecting a higher lipid fluidity in the immediate environment of the AChR.

Sunshine and McNamee (1994) also elaborated on the possibility that lateral phase separation occurs in the interaction of lipids with the AChR, such that the protein preferentially partitions into the more fluid domains. In the present work, laurdan emission spectra were recorded as a function of temperature (5°C to 50°C) using excitation wavelengths of 340 and 410 nm. Higher excitation GP values were obtained at 340 nm throughout the temperature range studied (not shown). This reinforces the view that the photoselection process, characteristic of phase coexistence, occurred neither in the native AChR-rich membrane nor in the liposomes prepared from their extracted lipids. It would thus appear that laurdan molecules lie in a single phase with some of the characteristics of the liquid phase (presence of a dipolar relaxation process) and other properties of the gel phase (high GP values). The description of the physical state of the AChR-lipid interface region can thus be further refined: whereas the results of Fig. 8 suggest the existence of a higher lipid rigidity in the immediate vicinity of the AChR than in the rest of the membrane, the experiments on spectrally resolved GP (Fig. 9) add that a single phase with intermediate properties occurs throughout the AChR-rich membrane, with the characteristics of the so-called liquid-ordered phase. Narayanaswami and McNamee (1993) suggested a more fluid lipid in the immediate vicinity of the AChR. The data on which their hypothesis is based were obtained, however, by using reconstituted AChR, with lipids exclusively in the gel phase, and below the phase transition temperature. The native AChR-rich membrane, as used in the present study, possesses a high cholesterol/phospholipid ratio and a high protein/lipid ratio (see review in Barrantes, 1989). An optimal fit of the energy transfer efficiency data indicated a value of 14 ± 1 Å for the minimum donor-acceptor distance, which corresponds roughly to the diameter of the first-shell protein-associated lipid. Combining this piece of information with the data stemming from the GP experiments leads to the interpretation that laurdan molecules within energy transfer distance (i.e., predominantly first-shell, protein-associated lipid) lie in a relatively more rigid environment than the bulk bilayer lipid region. Because laurdan essentially measures the degree of water penetration in the bilayer (Parasassi et al., 1991), this implies that the lipid mobility in the vicinity of

the protein is such that it allows for much lower penetration than the bulk, bilayer lipid. It is highly likely that the rigidifying effect exerted by the transmembrane portion of the AChR protein on boundary lipid is partly counterbalanced by the fluidizing effect of cholesterol located in this region, even though cholesterol in the nonvicinal, bulk lipid domain has the opposite (i.e., rigidifying) effect. The net effect would thus be an averaging one, diminishing abrupt transitions between the two lipid domains.

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REFERENCES

Barrantes, F. J. 1978. Agonist mediated changes of acetylcholine receptor in its membrane environment. *J. Mol. Biol.* 124:1–26.

Barrantes, F. J. 1982. Interactions between the acetylcholine receptor and the non-receptor, peripheral nu-peptide (M_r 43,000). *In* Neuroreceptors. F. Hucho, editor. W. de Gruyter, Berlin, New York. 315–328.

Barrantes, F. J. 1989. The lipid environment of the nicotinic acetylcholine receptor in native and reconstituted membranes. *Crit. Rev. Biochem. Mol. Biol.* 24:437–478.

Barrantes, F. J. 1993a. Structural-functional correlates of a rapid ligand-gated channel and its membrane environment: the nicotinic acetylcholine receptor. *FASEB J.* 7:1460–1467.

Barrantes, F. J. 1993b. The lipid annulus of the nicotinic acetylcholine receptor as a locus of structural-functional interactions. *In* Protein-Lipid Interactions. New Comprehensive Biochemistry, Vol. 26. A. Watts, editor. Elsevier, Amsterdam. 231–257.

Castuma, C. E., R. R. Brenner, E. A. DeLucca-Gattàs, S. Schreier, and M. T. Lamy-Freund. 1991. Cholesterol modulation of lipid-protein interactions in liver microsomal membrane: a spin label study. *Biochemistry.* 30:9492–9497.

Castuma, C. E., M. T. Lamy-Freund, R. R. Brenner, and S. Schreier. 1993. Lipid-protein interaction in a biological membrane: effect of cholesterol and acyl chain degree of unsaturation. *In* Protein-Lipid Interactions. New Comprehensive Biochemistry, Vol. 26. A. Watts, editor. Elsevier, Amsterdam. 87–106.

Chattopadhyay, A., and M. G. McNamee. 1991. Average membrane penetration depth of tryptophan residues of the nicotinic acetylcholine receptor by the parallax method. *Biochemistry.* 30:7159–7164.

Chong, P. L.-G. 1988. Effect of hydrostatic pressure on the location of PRODAN in lipid bilayers and cellular membranes. *Biochemistry.* 27: 399–404.

Chong, P. L.-G. 1990. Interactions of PRODAN and laurdan with membranes at high pressure. *High Pressure Res.* 5:761–763.

Criado, M., W. L. C. Vaz, F. J. Barrantes, and T. M. Jovin. 1982. Translational diffusion of acetylcholine receptor (monomeric and dimeric forms) of *Torpedo marmorata* reconstituted into phospholipid bilayers studied by fluorescence recovery after photobleaching. *Biochemistry.* 21:5750–5755.

East, J. M., O. T. Jones, A. C. Simmonds, and A. G. Lee. 1984. Membrane fluidity is not an important physiological regulator of the (Ca^{2+}-Mg^{2+})-dependent ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* 259: 8070–8071.

Fasman, G. D. 1976. Handbook of Biochemistry and Molecular Biology, Proteins, Vol. 1. CRC Press, Boca Raton, FL. 427.

Folch-Pi, J., M. B. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 266:497–509.

Fong, T. M., and M. G. McNamee. 1986. Correlation between acetylcholine receptor function and structural properties of membranes. *Biochemistry.* 25:830–840.

Förster, Th. 1948. Intermolecular energy migration and fluorescence. *Ann. Phys. (Leipzig).* 2:55–75.

Förster, Th. 1955. Fluoreszenz Organischer Verbindungen. Vandenhoch and Ruprecht, Göttingen.

Fung, B. K.-K., and L. Stryer. 1978. Surface density determination in membranes by fluorescence energy transfer. *Biochemistry.* 17: 5241–5248.

Gutiérrez-Merino, C. 1981. Quantitation of the Förster energy transfer for two-dimensional systems. II. Protein distribution and aggregation state in biological membranes. *Biophys. Chem.* 14:259–266.

Gutiérrez-Merino, C., F. Centeno, E. García-Martin, and J. M. Merino. 1994. Fluorescence energy transfer as a tool to locate functional sites in membrane proteins. *Biochem. Soc. Trans.* 22:784–788.

Gutiérrez-Merino, C., F. Munkonge, A. M. Mata, J. M. East, B. L. Levinson, R. M. Napier, and A. G. Lee. 1987. The position of the ATP binding site on the (Ca^{2+} + Mg^{2+})-ATPase. *Biochim. Biophys. Acta.* 897:207–216.

Gutiérrez-Merino, C., L. Pietrasanta, I. Bonini de Romanelli, and F. J. Barrantes. 1995. Preferential distribution of fluorescent phospholipid probes NBD-phosphatidylcholine and Rhodamine-phosphatidylethanolamine in the exofacial leaflet of acetylcholine receptor-rich membranes from *Torpedo marmorata. Biochemistry.* 34:4846–4855.

Homan, R., and M. Eisenberg. 1985. A fluorescence quenching technique for the measurement of paramagnetic ion concentrations at the membrane/water interface. Intrinsic and X537A-mediated cobalt fluxes across lipid bilayer membranes. *Biochim. Biophys. Acta.* 812:485–492.

Lakowicz, J. R. 1983. *In* Principles of Fluorescence Spectroscopy. Plenum Press, New York. 257–329.

Marsh, D., and F. J. Barrantes. 1978. Immobilized lipid in acetylcholine receptor-rich membranes from *Torpedo marmorata. Proc. Natl. Acad. Sci. USA.* 75:4329–4333.

Narayanaswami, V., and M. G. McNamee. 1993. Protein-lipid interactions and *Torpedo californica* nicotinic acetylcholine receptor function. 2. Membrane fluidity and ligand-mediated alteration in the accessibility of gamma subunit cysteine residues to cholesterol. *Biochemistry.* 32: 12420–12427.

Parasassi, T., F. Conti, and E. Gratton. 1986. Time-resolved fluorescence emission spectra of laurdan in phospholipid vesicles by multifrequency phase and modulation fluorometry. *Cell. Mol. Biol.* 32:103–108.

Parasassi, T., G. De Stasio, A. d'Ubaldo, and E. Gratton. 1990. Phase fluctuation in phospholipid membranes revealed by laurdan fluorescence. *Biophys. J.* 57:1179–1186.

Parasassi, T., G. De Stasio, G. Ravagnan, R. Rusch, and E. Gratton. 1991. Quantitation of lipid phases in phospholipid vesicles by the GP of laurdan fluorescence. *Biophys. J.* 60:179–189.

Parasassi, T., Di Stefano, M., Loeiro, M., Ravagnan, G., and Gratton, E. 1994. Influence of cholesterol on phospholipid bilayers phase domains as detected by laurdan fluorescence. *Biophys. J.* 66, 120–132.

Parasassi, T., M. Loiero, M. Raimondi, G. Ravagnan, and E. Gratton. 1993a. Absence of lipid gel-phase domains in seven mammalian cell lines and in four primary cell types. *Biochim. Biophys. Acta.* 1153:143–154.

Parasassi, T., R. Ravagnan, M. Rusch, and E. Gratton. 1993b. Modulation and dynamics of phase properties in phospholipid mixtures detected by laurdan fluorescence. *Photochem. Photobiol.* 57:403–410.

Rand, R. T. 1981. Interacting phospholipid bilayer: measured forces and induced structural changes. *Annual Rev. Biophys. Bioenerg.* 10, 277–314.

Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two-dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* 5:494–496.

Sunshine, C., and M. G. McNamee. 1994. Lipid modulation of nicotinic acetylcholine receptor function: the role of membrane lipid composition and fluidity. *Biochim. Biophys. Acta.* 1191:59–64.

Unwin, N. 1993. The nicotinic acetylcholine receptor at 9 Å resolution. *J. Mol. Biol.* 229:1101–1124.

Valenzuela, C. F., P. Weign, J. Yguerabide, and D. A. Johnson. 1994. Transverse distance between the membrane and the agonist binding sites on the *Torpedo* acetylcholine receptor: a fluorescence study. *Biophys. J.* 66:674–682.

Weber, G., and F. J. Farris. 1979. Synthesis and spectral properties of a hydrophobic fluorescent probe: 6-propionyl-2-dimethylaminonaphthalene. *Biochemistry.* 18:3075–3078.