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Preferential Distribution of the Fluorescent Phospholipid Probes NBD-Phosphatidylcholine and Rhodamine−Phosphatidylethanolamine in the Exofacial Leaflet of Acetylcholine Receptor-Rich Membranes from *Torpedo marmorata*†

C. Gutiérrez-Merino,‡ I. C. Bonini de Romanelli,§ L. I. Pietrasanta,§ and F. J. Barrantes*,§

Instituto de Investigaciones Bioquímicas, Universidad Nacional del Sur and Consejo de Investigaciones Científicas y Técnicas, C.C. 857, 8000 Bahía Blanca, Argentina, and Departamento de Bioquímica y Biología Molecular y Genética, Facultad de Ciencias, Universidad de Extremadura, Badajoz, Spain

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ABSTRACT: The distribution of the two fluorescent phospholipid analogs across acetylcholine receptor (AChR)-rich membranes from *Torpedo marmorata* has been studied by a combination of nonradiative fluorescence resonance energy transfer using fluorescent lipid probes and quenching of their fluorescence with Co^{2+} and 2,4,6-trinitrobenzenesulfonic acid. The fluorescent lipid analogs were supplied to the AChR-rich membrane or liposome suspension by simply injecting ethanol solutions of the probes into the medium. The efficiency of the fluorescence energy transfer between NBD-labeled phosphatidylcholine and rhodamine-labeled ethanolamine glycerophospholipids was measured in model membranes prepared in such a way that the probes could be targeted at the same or opposite halves of the bilayer, and the results were compared with those obtained for native AChR-rich membranes. It is shown that NBD-PC and Rho-PE can be efficiently (95%) incorporated into AChR-rich membranes and liposomes. On the basis of the comparison with model liposomes, the energy transfer experiments suggest a preferential exofacial location of the parental phospholipids in the native AChR-rich membrane. Fluorescence quenching with Co^{2+} and TNBS showed these two phospholipid analogs to be located predominantly in the outer leaflet of the bilayer in AChR-rich membranes. From the Co^{2+} quenching of the lipid analogs, it was also possible to calculate the surface potential of the outer leaflet of the membrane as being on the order of −15 mV.

The nicotinic acetylcholine receptor (AChR)[1] is an oligomeric transmembrane glycoprotein. It occurs at very high densities in the postsynaptic membrane [20000−50000 particles/μm^2, depending on the biological source; see Popot and Changeux (1984) for a review]. Another relevant structural characteristic of this protein is its vectorial sidedness with respect to the membrane, with a bulkier domain carrying the agonist recognition site oriented toward and protruding into the extracellular space [see Unwin (1993)]. Such asymmetry of the AChR along its main axis perpendicular to the plane of the membrane raises the question of whether the lipid constituents of the postsynaptic membrane are also distributed asymmetrically between the two halves of the bilayer (Barrantes, 1989, 1993). The question concerns both phospholipid head group and fatty acyl chain topography.

Chemical probes under nonpenetrating conditions [e.g., see Op den Kamp (1979); Litman, 1982], limited hydrolysis of lipids by phospholipases (e.g., Op den Kamp, 1979; Roelofsen, 1982), and fluorescence methods [for reviews, see Schroeder (1988) and Devaux (1991)] have been useful in establishing that the lipid classes present in many biological membranes tend to be distributed asymmetrically between the outer and inner leaflets of the bilayer (Rothman & Lenard, 1977; Higgins & Hutson, 1986). Compositional asymmetry has also been experimentally determined in some biomembranes by chemical and enzymatic methods [see, for examples, reviews by Etemadi (1980) and van Deenen (1981)]. In the erythrocyte, phosphatidylcholine (PC) and sphingomyelin principally occur in the exofacial leaflet [76% of the PC; see Op den Kamp et al. (1985)], whereas the aminophosphoglyceride phosphatidylethanolamines (PE) and phosphatidylserine (PS) are mainly cytofacial (Op den Kamp, 1979). The asymmetrical distribution appears to have physiological significance: in platelets, lipid modification alters membrane curvature (Daleke & Huestis, 1985); in erythrocytes, the appearance of PS on the outer leaflet has been interpreted as a signal for the removal of the erythrocyte from the bloodstream (Tanaka & Schroit, 1983).

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* Author to whom correspondence should be addressed.

‡ Universidad de Extremadura.

§ Universidad Nacional del Sur and Consejo de Investigaciones Científicas y Técnicas.

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[1] Abbreviations: AChR, acetylcholine receptor; DOPC, dioleoylphosphatidylcholine; LUV(s), large unilamellar vesicle(s); PC, phosphatidylcholine; Phl, phospholipids; PS, phosphatidylserine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-morpholinopropanesulfonic acid; *N*-Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; 6-NBD-PC, 1-oleoyl-2-[6-[(7-nitro-2, 1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine; 12-NBD-PC, 1-oleoyl-2-[12-[(7-nitro-2, 1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylcholine; NBD-PE, *N*-(7-nitro-2, 1,3-benzoxadiazol-4-yl)dipalmitoylphosphatidylethanolamine; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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After we examined in some detail both the composition (Rotstein et al., 1987a) and metabolism (Rotstein et al., 1987b; Bonini de Romanelli et al., 1987) of the electrocyte lipid constituents, it was important to ascertain their topography in AChR-rich membranes. Chemical derivatization of AChR membrane aminophospholipids with trinitrobenzenesulfonate (TNBS) and hydrolysis by phospholipase C have been used to learn about the location of some phospholipids in this membrane (Bonini de Romanelli et al., 1990). However, both treatments fall short of providing information on the topography of lipids within the same leaflet of the bilayer, either with vesicles of membrane fragments or with cells. Other methodological approaches therefore are required to tackle this question. Toward this end, we have studied the feasibility of employing fluorescent phospholipid analogs in AChR membranes of the two major lipids in these membranes, PC and PE. On the basis of fluorescence energy transfer and quenching studies undertaken on these and model membranes, a preferential exofacial location in the native AChR-rich membrane can be inferred. Such studies also provide an estimation of the surface potential of the AChR-rich membrane relative to that of vesicles prepared from the endogenous phosphatidylcholine (PC) extracts of this membrane.

MATERIALS AND METHODS

Materials. Electric fish (*Torpedo marmorata*) were obtained from the Biological Marine Station at Arcachon, France. NBD-PC and *N*-Rho-PE were purchased from Avanti Polar Lipids (Birmingham, AL). *Bacillus cereus* phospholipase C (EC 3.1.4.3), DOPC, MOPS, *N*-eosin-*N'*-phosphatidylethanolamine, EDTA, 2,4,6-trinitrobenzenesulfonate, and $CoCl_2$ were purchased from Sigma Chemical Co. (St. Louis, MO). Sepharose 4B was from Pharmacia.

Purification and Subfractionation of AChR-Rich Membrane Fragments. Membranes were prepared according to Barrantes (1982). Typically, specific activities on the order of 1.0−2.5 nmol α-bungarotoxin binding sites/mg of protein were obtained. The orientation of AChR in vesicles was determined by measuring 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 (Hartig & Raftery, 1979). A subpopulation of totally sealed vesicles, as defined by their ability to retain Cs^+ in their interior (Sachs et al., 1982), was prepared from the AChR-rich membrane fraction. Fluorescence experiments were also conducted using this membrane preparation.

Measurement of AChR-Rich Membrane Integrity by Phospholipase C Hydrolysis. The goal of the subcellular fractionation technique developed in our laboratory (Barrantes, 1982) for the preparation of AChR-rich vesicles was maximal preservation of vesicle integrity. The morphological criteria used then—electron microscope examination—are complemented here with the technique subsequently developed by Bonini de Romanelli et al. (1990). Basically, phospholipase C hydrolysis was carried out using the enzyme from *B. cereus* without further purification. The incubation medium consisted of 5 mM Tris-HCl buffer (pH 7.4), 0.2 M sucrose, 0.3 M NaCl, and 0.2 mM EGTA (Michaelson et al., 1983). The reaction, carried out at 25 °C, was started by addition of the enzyme (0.25 unit/mL) to the membrane suspension. Aliquots containing about 2.5 μL of lipid phosphorus were taken at various intervals, and the reaction was stopped by adding chloroform−methanol (1:2, v/v), a solvent mixture in which the enzyme showed no activity in control experiments. In this series of experiments, lipid extracts were subsequently prepared and partitioned according to the procedure of Bligh and Dyer (1959) and assayed for inorganic phosphorus content as described by Rouser et al. (1970).

Preparation of Endogenous Lipid Extracts and PC Fractions Thereof and Their Phospholipid Analysis. Endogenous lipids were extracted from *T. marmorata* AChR-rich membranes by the method of Folch et al. (1957). Two successive monodimensional chromatography steps were used to isolate phospholipid fractions from these lipid extracts using thin-layer chromatography on 500 μm thick silica gel H plates. Fractions were eluted according to Arvidson (1968), and phospholipids were assayed as described by Rouser et al. (1970). The phosphatidylcholine fraction was used to prepare large unilamellar vesicles, as described in the following.

NBD-Phospholipid and N-Rho-PE Incorporation into AChR-Rich Membranes. AChR-rich membranes (50 μg of protein/mL, about 1300 α-toxin sites/mg of protein) were suspended in 1 mL of 50 mM MOPS buffer containing 100 mM NaCl and 1 mM EDTA (pH 7.4) and supplemented with 1.5 mol % 6-NBD-PC, 12-NBD-PC, or NBD-PE (0.41 nmol, 1.5 mol % with respect to total membrane lipids). Membranes were incubated under constant stirring for 20 min at room temperature and then centrifuged at 120000*g* for 30 min in a Ti-80 rotor using a Beckman LS-85 ultracentrifuge. The supernatant was discarded, and the pellet was resuspended in 1.5 mL of MOPS buffer. These NBD-phospholipid-labeled membranes were then supplemented with the appropriate concentration of *N*-Rho-PE (0.25−3 mol %) in ethanol and incubated for 20 min at room temperature. Conversely, *N*-Rho-PE-labeled membranes were supplemented with NBD-phospholipids in the same molar range. Lipid uptake in the presence of 1% Triton X-100 was determined by measuring the relative NBD and *N*-Rho fluorescence in aliquots of the AChR membrane suspension.

Preparation of Model Membranes Doped with Fluorescent Probes. (I) Symmetric Vesicles. (a) Vesicles Containing N-Rho-PE and NBD-Phospholipids in Both Leaflets. Large unilamellar vesicles made from PC purified from lipid extracts obtained from *T. marmorata* AChR membranes [PC constitutes the predominant phospholipid in this membrane; see Rotstein et al. (1987a,b)] or DOPC (Connor & Schroit, 1987) and containing the indicated amounts of symmetrically distributed *N*-Rho-PE and 6-NBD-PC, 12-NBD-PC, or NBD-PE were prepared by resuspending mixed, dried lipids in 20 mM HEPES buffer (pH 7.4) containing 145 mM NaCl, 5 mM KCl, and 10 mM glucose to give a final concentration of 1 mg of lipid/mL.

(b) Vesicles Containing N-Rho-PE and NBD-Phospholipids in Their Outer Leaflet. Insertion of the fluorescent lipids into the outer leaflet only was accomplished by injecting, under constant mixing, appropriate amounts of NBD-phospholipids in ethanol (final ethanol concentration <1%) into preformed large unilamellar vesicles, following the procedures of Kremer et al. (1977) and Wolf (1985). The suspension was dialyzed against 4 L of HEPES buffer for 24 h, and the procedure was repeated with *N*-Rho-PE.

(II) Preparation of Asymmetric Vesicles (i.e., Containing One Probe Preferentially in One Leaflet and the Other Preferentially in the Opposite Leaflet). Large unilamellar

vesicles were initially prepared as described earlier from AChR-rich membrane PC extracts containing a fixed amount of NBD-phospholipids. The formation of asymmetric vesicles basically followed the procedure of Connor and Schroit (1987). The large unilamellar vesicles, containing NBD-phospholipids in both leaflets of the bilayer, were used as fluorescent probe donors as follows. Large unilamellar vesicles were mixed with a 5-fold excess (w/w) of a homogeneous population of small unilamellar acceptor vesicles prepared according to Struck and Pagano (1980). These acceptor vesicles were made from PC extracts prepared from *Torpedo* AChR-rich membranes, presized on Sepharose 4B. Large unilamellar donor and small unilamellar acceptor vesicles were incubated at 4 °C for 24 h. The mixed vesicle populations were then separated by chromatography on a Sepharose 4B column (1 × 40 cm). Fractions were collected and monitored for NBD fluorescence. The large unilamellar vesicles eluted with the void volume, while the small unilamellar vesicles (containing NBD-phospholipids exchanged from the outer leaflet of the large unilamellar donor vesicle) eluted as a distinct, included peak. The presence of NBD-phospholipids only in the inner leaflet was verified by analysis of NBD-phospholipid distribution in the excluded (large unilamellar vesicles) and included (small unilamellar vesicles) peaks, which revealed that the fluorescent lipid was distributed equally between the two vesicle populations (Pagano et al., 1981).

Fluorescence Measurements. Resonance energy transfer efficiency (E) was measured in various types of membrane systems by assessing the degree of donor (NBD-phospholipid) quenching. The analysis of the data was carried out as indicated in Gutiérrez-Merino et al. (1987, 1989). Briefly, the overall energy transfer of the ensemble 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., $\langle k \rangle = \sum k_i$, where the rate of transfer for pair i separated by a distance r_i is k_i, provided that the diffusion rate of donor and acceptor is much slower than the lifetime of the excited state of the donor. This expression has been used to measure relative positions of protein and lipid in a mixture. 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, for example, Gutiérrez-Merino (1981a,b) and Gutiérrez-Merino et al. (1987, 1989)]. The overall efficiency of the fluorescence energy transfer, $\langle E \rangle$, can be written as

$$\langle E \rangle = \langle k \rangle / k_o + \langle k \rangle \quad (1)$$

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%. When necessary, R_o was experimentally determined as indicated by Gutiérrez-Merino et al. (1987, 1989). The analytical approach developed in the latter papers was used to calculate the average rate of Förster energy transfer as a function of the position of the donor with respect to acceptors, all of which are located in the membrane. One necessary assumption is that the number of acceptor molecules is much greater than the number of donor molecules. A second assumption implies viewing the distribution of both donor and acceptor molecules as a continuum of disks of different sizes. Thirdly, it is necessary to assume random mixing of labeled and unlabeled lipid molecules in the membrane.

Experimentally, measurements of the extent of quenching of donor fluorescence by Förster energy transfer in the absence and presence of increasing concentrations of acceptor were taken using an SLM 4800 spectrofluorimeter and 10 × 10 mm quartz cuvettes. Other steady state fluorescence measurements were carried out using this instrument. Emission spectra were corrected for wavelength-dependent distortions and dilution effects. Detergent-mediated quenching effects (Tanaka & Schroit, 1983) were also taken into account. Control experiments were carried out to discard the possibility that the absorption and/or emission spectra of NBD- and Rho-tagged lipids were affected by the addition of 1% Triton X-100. The results clearly showed that the presence of 1% Triton did not significantly alter either the absorption or the emission spectra. In particular, there was no noticeable shift in the absorption and emission peaks or in the fluorescence quantum yield. It is to be noted that Wolf et al. (1992), using an approach similar to ours, but with large dioleoylphosphatidylethanolamine and sphingosylphosphorylcholine vesicles, and Connor and Schroit (1987), with human red cells, did not notice any alteration in the spectral properties of NBD-labeled lipids in the presence of 1% Triton X-100.

Inner filter effects were negligible, except for the quenching experiments described later using Co^{2+}, in which case the corrections described by Lakowicz (1983) and Homan and Eisenberg (1985) were applied. 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, in all experiments, changes in the turbidity of the samples were kept below 0.05 OD unit (note that labeled lipids were added at a low molar ratio, <4%), and on average only about 50 μg of AChR membrane protein/1.5 mL (approximately 27.5 nmol of phospholipids/1.5 mL) was used in each cuvette. In addition, in fluorescence energy transfer measurements samples were brought to a final concentration of 1% Triton X-100, thus making the fluorescence intensity of the donor an internal control of the sample concentration, since the molar fraction of the donor labeled lipid was kept constant. This also enabled us to demonstrate that quenching was due to energy transfer to lipids labeled with acceptor fluorophores. The quenching of donor fluorescence by energy transfer was calculated from donor quenching before the addition of 1% Triton X-100. When appropriate, corrections for changes in membrane concentration were made. Values of less than 10% were usually obtained within a given experimental series. Theoretical plots of energy transfer efficiency were calculated according to Gutiérrez-Merino (1981a,b) by assuming an average area per lipid molecule of about 0.75 nm (Rand, 1981).

For the Co^{2+} quenching experiments, model and AChR-rich membranes labeled with the fluorescent lipid analogs as described earlier were supplemented with aliquots of 1.0 M $CoCl_2$ in the presence and absence of 0.1% saponin. At this concentration of the permeabilizing agent, no changes in the absorption and/or fluorescence properties of the NBD- or Rho-labeled lipids were observed. In the case of TNBS quenching experiments, membranes doped with the lipid fluorescent probes were first taken to the appropriate pH by

centrifugation at 120000*g* for 30 min and resuspension in 435 mM $NaHCO_3$ buffer (pH 8.5) and titrated with increasing concentrations of TNBS at 4 °C (nonpenetrating conditions) or 37 °C (penetrating conditions).

Fluorescence lifetime determinations were made using the same SLM Model 4800 spectrofluorimeter by exciting samples with sinusoidal light modulated at a known frequency and measuring the demodulation or the shift in the phase angle of the emitted light. Polystyrene beads were used as a scattering reference for excitation modulation. Steady state intensities of polystyrene bead scatter and lipid probe fluorescence were matched by adjusting the concentration of the former.

Estimation of Surface Charge. The surface potential sensed by an ion, ϕ, is related to the surface density of charge, σ, at the lipid−water interface by the equation (Lee, 1977)

$$\phi = (2kT/z)\ \mathrm{arcsinh}(\sigma/B\mu^{1/2}) \quad (2)$$

where $B = (8\epsilon RT)^{1/2}$; ϵ is the dielectric constant relative to vacuum, μ is the ionic strength, and z is the net charge of the ion.

The value of σ can also be calculated from the composition and distribution of phospholipids between the two leaflets of the membrane bilayer (Fernández, 1981):

$$\sigma = -P_h e/100A_m \quad (3)$$

where e is the electron charge and A_m is the average area of a phospholipid, expressed as a percent of unit electronic charge per phospholipid. The value of P_h, the percentage of ionized phospholipid, can be estimated by averaging the net charge of different phospholipids in the membrane and their pK values [see, for example, Toccane and Teissié, 1990)].

RESULTS

Sidedness and Integrity of AChR-Rich Membranes. Phosphatidylcholine (PC) followed by ethanolamine glycerophospholipids (PE + plasmenylethanolamine) and phosphatidylserine (PS) together constitute more than 85% of all phospholipids in AChR membranes (Rotstein et al., 1987a; Bonini de Romanelli et al., 1987). Given the aim of the present work, it was necessary to establish the sidedness of the membrane vesicles and their integrity. The vectorial orientation of the AChR protein provides a useful landmark toward this goal. The proportion of right side out AChR vesicles (Hartig & Raftery, 1979) amounted to 82 ± 7% (n = 3).

The vesicle integrity of the membrane preparation employed in the present study has been documented (Barrantes, 1982). An additional criterion was applied in the present work, using phospholipase C hydrolysis of AChR membrane phospholipids (Bonini de Romanelli et al., 1990). The enzyme reacted with an overall rate of about 400 nmol of lipid phosphorus/min for the initial phase of the reaction; in the succeeding 3−10 min interval, the rate diminished to about 50 nmol min^{-1}, in agreement with earlier results from this laboratory showing that about half of the PC, PE, PS, and total phospholipids were digested at 25 °C with half-times of 1.7, 1.6, and 1.8 min, respectively. The lack of digestion of other lipids during the initial phase of phospholipase action is consistent with the interpretation that it is only after the hydrolysis of about half of their lipids that AChR-rich membranes are disrupted and cytoplasmic-facing lipids thus become accessible to the enzyme. This serves as a measure of vesicle integrity. The present results are also in agreement with those obtained with TNBS chemical derivatization of the amino phospholipids in AChR-rich membranes (Bonini de Romanelli et al., 1990). In order to further validate the conclusions from our fluorescence experiments, AChR-rich membranes were further fractionated following the procedure of Sachs et al. (1982) in order to separate sealed from leaky vesicles. A series of fluorescence experiments carried out on the subpopulation of sealed vesicles gave essentially the same results as those obtained with the native AChR-rich membrane prepared according to Barrantes (1982).

Fluorescence Properties of Fluorescent Phospholipid Analogs in Native AChR-Rich Membranes. Addition of the fluorescent lipid probes in ethanol solution to a suspension of AChR-rich membranes resulted in their efficient incorporation, as measured by the fluorescence remaining in the supernatant after centrifugation at 120000*g*: less than 5% of the total fluorescence was found in this fraction in all cases. Upon incorporation into the AChR-rich membrane, none of the probes studied (NBD, Rho, or fluorescein derivatives; not shown) showed any major alteration of their excitation or emission bands, in agreement with earlier observations of similar probes with other biological membranes (Gutiérrez-Merino et al., 1987; Connors & Schroit, 1987) and with model liposomes (Fung & Stryer, 1978; Gutiérrez-Merino et al., 1987; Chattopadhyay & London, 1988). In addition, the binding of [^{125}I]-α-bungarotoxin was not altered by the incorporation of any of the fluorescent probes.

In all cases, however, the polarization of fluorescence of the membrane-associated probes was found to be higher than that of the fluorescent labels free in solution. This alone shows fluorescence polarization measurements to be a good tool for critically estimating the incorporation of lipid derivatives into the membrane. Furthermore, the value of fluorescence polarization attained by *N*-Rho-PE in AChR-rich membranes (P = 0.24−0.25) was significantly higher than that measured in liposomes prepared from synthetic PC (P = 0.12−0.15). We have observed similar results with *N*-fluoresceinyl-PE (not shown).

Determination of NBD-Phospholipid/N-Rho-PE Sidedness in Model Lipid Vesicles and in the Native AChR Membrane. The dimensions of a lipid bilayer, 4−5 nm (Jain & Wagner, 1980; Herbette et al., 1985), are in the same range as R_0, the theoretical distance at which a 50% resonance energy transfer has been found to occur between various pairs of donor and acceptor fluorescent molecules currently used in biophysical studies of biological membranes, such as [(dimethylamino)-naphthalene-5-sulfonyl]-PE/*N*-eosin-*N'*-(phosphatidylethanolamine)thiourea (Fung & Stryer, 1978), fluorescein/Rho (Gutiérrez-Merino et al., 1987), or NBD/Rho (Connors & Schroit, 1987; Wolf et al., 1992; this work). It therefore can be predicted that the efficiency of fluorescence energy transfer between donor and acceptor labeled lipids located in opposite leaflets of the membrane bilayer will be considerably lower than that between donor and acceptor molecules present in the same face of the membrane.

Table 1: Characteristic Parameters of the Donor/Acceptor Pairs Employed in This Study

donor/acceptor pair[a]	extinction coefficient[b]	Q_D	J (cm^3/M)	R_o[c] (nm)
NBD-Phl/*N*-Rho-Phl	6.83×10^4	0.4	3.47×10^{-13}	5.58
NBD-Phl/Co^{2+}	6.5×10^4	0.4	2.34×10^{-18}	0.80

[a] A value of 1.30 was taken as the refractive index corresponding to a dilute solution, and the value of k^2 was set at $^2/_3$, i.e., random orientation between donor and acceptor molecules has been assumed (see text). [b] Measured at the maximum absorption wavelength. [c] The distance R_o is calculated as in Lakowicz (1973) as $R_o = 9.79 \times 10^3(k^2 J Q_D n^{-4})^{1/6}$, where J is the overlap integral (cm^3/M), Q_D is the donor quantum yield in the absence of acceptor, and n is the refractive index of the medium. k^2 is the orientation factor that accounts for the relative orientation of the donor emission and acceptor absorption transition dipoles. In systems where energy transfer takes place from one donor to many acceptors, with donor and acceptor bound to different molecules, the assumption of random orientation is amply justified [see, for example, Gutiérrez-Merino et al. (1981a)].

Following the protocols outlined under Materials and Methods, different types of labeled lipids were incorporated into model vesicles of defined lipid composition and into AChR-rich native membranes. The average efficiency of energy transfer between donor and acceptor probes incorporated into the membranes, $\langle E \rangle$, was calculated using the equation:

$$\langle E \rangle = 1 - F/F_D \quad (4)$$

where F and F_D are the steady state fluorescence intensity of the donor lipid in the presence of a given concentration of acceptors and that measured in the absence of energy transfer [e.g., after the addition of a solubilizing concentration of detergent (Fung & Stryer, 1978)]. The labeled lipids used in this study carry the NBD group (donors) labeled at different positions (head group, C_6, C_{12}), and *N*-Rho-PE is used as the acceptor in all cases. A value of R_o of 5.58 nm was calculated for this pair using the experimentally determined parameters listed in Table 1, which were obtained from the corrected emission spectra of NBD-labeled phospholipids and the absorption spectrum of *N*-Rho-PE. This value of R_o is in full agreement with the values produced by Connors and Schroit (1987) for this donor/acceptor pair and by Wolf et al. (1992) (5.5−5.6 nm) for NBD-PE/*N*-Rho-PE in different experimental systems.

Fluorescence energy transfer experiments were then undertaken using various protocols. In a first series of experiments, the efficiency of fluorescence energy transfer of AChR membranes containing constant concentrations of donor fluorescent probe (1.5 mol % NBD-labeled phospholipid) and increasing concentrations of acceptor (*N*-Rho-PE, 0.25−3.0 mol %) was measured and compared with the efficiency of energy transfer of the same pair of probes in model lipid membranes, i.e., vesicles prepared from a PC fraction obtained from *Torpedo* AChR-rich membrane lipid extracts or synthetic DOPC liposomes, respectively (Figures 1 and 2). As expected for a constant molar ratio of the donor NBD-tagged lipid to total phospholipids, ⟨E⟩ varied as a function of the *N*-Rho-PE acceptor surface density.

The efficiency of resonance energy transfer between 6- or 12-NBD-PC and *N*-Rho-PE in liposomes prepared from PC extracts from *Torpedo* AChR-rich membranes was found to be high, reaching values of about 90% for acceptor/donor ratios higher than unity (i.e., for molar acceptor/total lipid ratios of 0.02 or higher), when the model membranes were prepared such that both probes were predominantly in the outer leaflet of the bilayer (Figure 2). Energy transfer efficiency in lipid vesicles prepared with a PC fraction obtained from *Torpedo* AChR-rich membrane endogenous lipid extracts or synthetic DOPC, and with the fluorescent probes in *opposite* leaflets of the bilayer (i.e., asymmetric vesicles), reached much lower values (less than 40% at

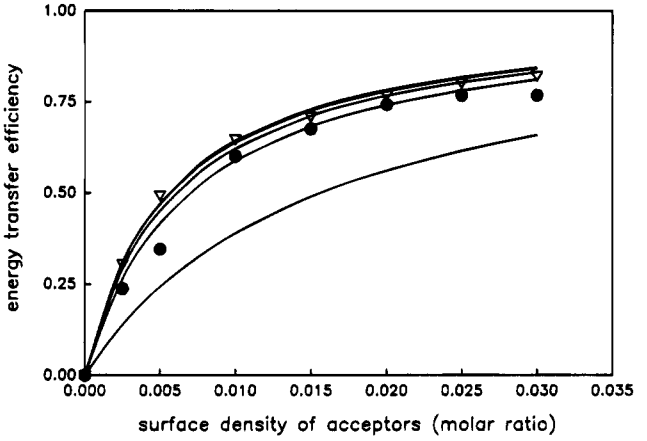

FIGURE 1: Efficiency of resonance energy transfer and calculated distances between donor/acceptor pairs of fluorescent lipid analogs in native AChR-rich membranes as a function of the surface density of energy transfer acceptors. A constant concentration of the fluorescent donor (1.5% NBD-PE, ▽; 1.5% 12-NBD-PC, ●) was incorporated into the membranes, and then the donor fluorescence was titrated with increasing concentrations of the fluorescent acceptor (*N*-Rho-PE, 0.25−3.0 mol %). The curves (solid lines) correspond to theoretical fits with the donor−acceptor minimal distances (from top to bottom) of 0 and 1 nm (overlapping) and 2, 3, and 5.3 nm, respectively. The concentration of protein in AChR-rich membranes was kept at about 35 μg/mL; the phospholipid concentration was about 18−20 μM in these and other experiments shown in this paper.

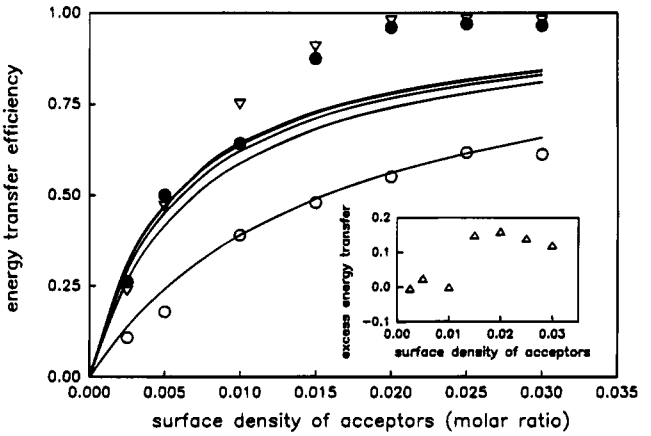

FIGURE 2: Efficiency of resonance energy transfer and calculated distances between donor/acceptor pairs of fluorescent lipid analogs in model lipid membranes as a function of the surface density of energy transfer acceptors (*N*-Rho-PE). Model liposomes prepared from the PC fraction from *T. marmorata* AChR-rich membranes were supplemented with a constant concentration of the fluorescent donor (1.5 mol % 12-NBD-PC in the outer leaflet, ●; 12-NBD-PC in opposite leaflets, ○; NBD-PE in the external leaflet, ▽). Membranes were titrated with increasing concentrations of the fluorescent acceptor (*N*-Rho-PE, 0.25−3.0 mol %). The curves correspond to theoretical fits with the donor−acceptor distances (from top to bottom) of 0 and 1 nm (overlapping) 2, 3, and 5.3 nm, respectively. Inset: Excess energy transfer versus surface density of acceptor fluorophores (see text).

N-Rho-PE/12-NBD-PC ratios of 1.0; Figure 2). Similar values were obtained with 6-NBD-PC (not shown). Replacement of the NBD-PC labeled at carbon 12 by an NBD-PE probe having the fluorophore in its polar head resulted in higher energy transfer efficiency (Figure 2). Likewise, model vesicles with NBD-PE as the donor were prepared, and the patterns were similar to those found with 6-NBD-PC.

The experimental results corresponding to the 12-NBD-PC/*N*-Rho-PE pair obeyed the theoretical predictions for fluorophores lying on two planes separated by about 5.5 nm. This is compatible with the expected values for the lipid bilayer thickness, assuming that the NBD moiety tends to position itself close to the lipid−water interface. This latter assumption turned out to be fully consistent with the present fluorescence quenching data, with the recent results of Wolf et al. (1992), with the location of the NBD group of NBD-labeled lipids in the proximity of the lipid−water interface (Chattopadhyay, 1990), and with other conclusions on the ionization properties of the NBD group covalently bound to PC or PE (Chattopadhyay & London, 1987, 1988). However, for the case of both donor and acceptor located in the *same* leaflet of the bilayer, the fit of ⟨E⟩ between NBD-phospholipids and *N*- Rho-PE to the theoretical prediction is not as good as in the case of donor/acceptor pairs located in *opposite* membrane leaflets (Figure 2). In particular, at high surface density of acceptors, the experimental data exhibit a clear tendency to deviate toward higher values of energy transfer than those expected. The excess energy transfer is depicted graphically in the inset to Figure 2. This effect has also been noticed with fluorescein-PE/*N*-Rho-PE (Gutiérrez-Merino et al., 1987) and indicates that the aromatic probes exhibit a tendency to interact in the lipid bilayer, thus leading to a nonrandom distribution of the probes at the membrane surface.

In contrast, in native AChR-rich membranes the observed dependence of energy transfer efficiency between lipid analogs closely follows that of the theoretical expectations (cf. Figure 1). The latter series of experiments thus strongly suggests that the fluorescent lipid analogs are located in the same leaflet in AChR membranes; any conclusion regarding the other two possibilities (the NBD-phospholipid in the outer leaflet and the *N*-Rho-phospholipid in the inner one or vice versa) is precluded by experimental uncertainties. Comparison with model membranes did already suggest that both probes are predominantly in the outer leaflet of the bilayer in AChR-rich membranes.

Refinement of the Location of NBD-PC and Rho-PE in AChR Membranes by Cobaltous Ion and TNBS Quenching Experiments. The localization of *N*-Rho-PE and 6-NBD-PC incorporated into AChR-rich membranes and model lipid membranes was further studied by Co^{2+} and TNBS quenching experiments. The fluorescence of *N*-Rho-PE, NBD-phospholipids, or NBD-PE was quenched by Co^{2+} in the presence and absence of 0.1% saponin (Figure 3 and Table 2), a membrane-permeabilizing agent, and analyzed according to the Stern−Volmer equation modified by Lehrer (Figure 3). From such analysis, the fraction of the total fluorescent lipid available to Co^{2+} (f_a) and the quenching constant (K_{SV}) were determined in model and AChR membranes. The results also show the efficiency of the selective labeling of the target membrane leaflet in model membranes (Figure 3).

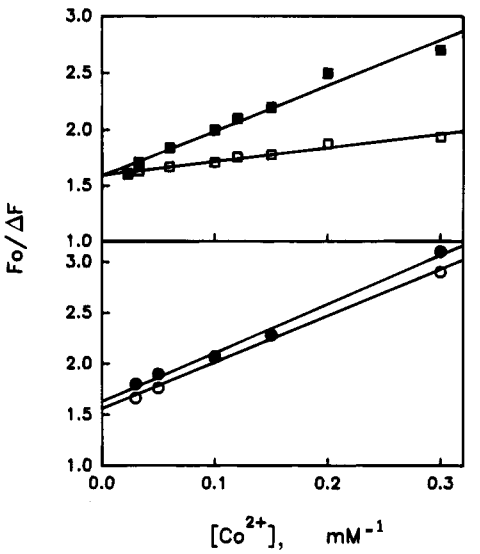

FIGURE 3: Upper panel: Modified Stern−Volmer plot of Co^{2+} quenching of *N*-Rho-PE in native (□) and 0.1% saponin-treated AChR-rich membranes (■). Lower panel: Liposomes prepared from *Torpedo* AChR-rich membrane endogenous PC extracts doped with NBD-PE in the exofacial leaflet before (●) and after 0.1% saponin treatment (○).

Table 2: Quenching of NBD-PC and *N*-Rho-PE Fluorescence by Co^{2+} in AChR-Rich and Model Lipid Membranes[a]

	sample	f_a	$1/K_{SV}$ (mM)
6-NBD-PC			
DOPC liposomes	symmetric	0.50 ± 0.06	32.1 ± 9.8
	outer leaflet	0.62 ± 0.02	11.5 ± 4.0
PC liposomes	symmetric	0.52 ± 0.07	14.1 ± 6.8
	outer leaflet	0.74 (n = 2)	11.6
endogenous PC	symmetric	0.59 ± 0.04	10.9 ± 1.6
	outer leaflet	0.71 ± 0.01	6.5 ± 0.6
AChR-rich membranes		0.68 ± 0.01	2.5 ± 0.3
N-Rho-PE			
DOPC liposomes	symmetric	0.50 ● 0.04	29.3 ± 1.3
	outer leaflet	0.67 ± 0.05	10.8 ± 1.9
PC liposomes	symmetric	0.45 ± 0.03	32.0 ± 2.4
	outer leaflet	0.74 ± 0.05	14.3 ± 1.5
endogenous PCs	symmetric	0.56 ± 0.01	17.0 ± 3.4
	outer leaflet	0.71 ± 0.01	7.1 ± 0.1
AChR-rich membranes		0.63 ± 0.02	2.7 ± 1.1

[a] See the conditions for obtaining model lipid membranes under Materials and Methods. f_a is the fraction of the total fluorescent probe population accessible to the quencher; K_{SV} is the apparent quenching constant derived from the Stern−Volmer relationship modified by Lehrer (1977): $F_o/\Delta_F = [f_a K_o\,[Q]]^{-1} + f_a^{-1}$, where F_o is the fluorescence intensity in the absence of quencher, Δ_F is the difference in fluorescence intensity in the absence and presence of quencher, [Q] is the concentration of quencher, and $1/K_{SV}$ is the quencher concentration at which 50% of the intensity is quenched. Whenever values ± SD are given, these are the average of at least three different experiments.

The fraction of fluorophores at infinite quencher concentrations was about 68% for 6-NBD-PC in the AChR-rich membrane, augmented to about 71% in the presence of 0.1% saponin and in the 3−45 mM $CoCl_2$ concentration range (Table 2). In the case of 12-NBD-PC, the figures were 65% and 80% (not shown). With *N*-Rho-PE, the similarity of the value of f_a in the native AChR-rich membrane (63 ± 2%) with that in the presence of 0.1% saponin (63 ± 6%) indicated even more dramatically that the probe is predominantly exposed to the quencher and that permeabilization did not alter its location. Furthermore, the values of f_a are

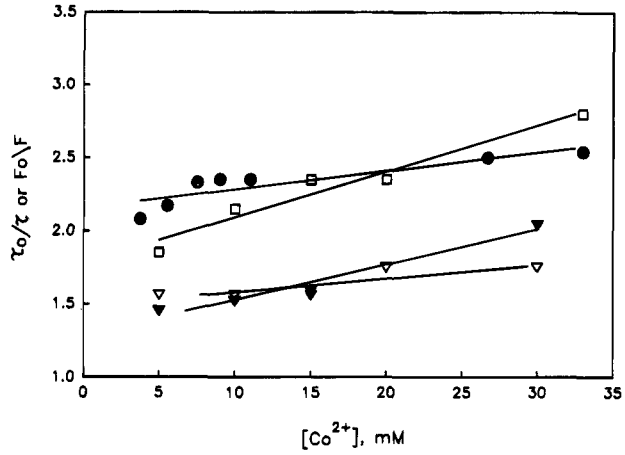

FIGURE 4: Plots of ratios of total fluorescence intensity (F_o/F) and of nanosecond fluorescence lifetime (τ_o/τ) versus cobalt concentration in AChR-rich membranes: (●) F_o/F for *N*-Rho-PE; (□) F_o/F for NBD-PC; (▼) τ_o/τ for NBD-PC; ▽ τ_o/τ for *N*-Rho-PE. Notice that F_o/F values are consistently higher than those of τ_o/τ.

similar to those observed for model membranes prepared such that the probe is located exclusively in the outer leaflet (Table 2). Upon membrane permeabilization with saponin, f_a values attained levels comparable to those obtained with the fluorophore in the outer leaflet. Thus, the *native AChR membrane resembled the model system with the probe incorporated in the outer leaflet only.* Permeabilization brings the fraction of available fluorophores in the AChR membrane to similar levels, indicating equivalence in the intrinsic accessibility to the quencher: once permeabilized, a small proportion of the probes, presumably located in the inner leaflet, can be reached by the quencher. The simplest explanation for the increase in K_{SV} observed upon permeabilization with 0.1% saponin in the AChR-rich membrane, but not in the synthetic liposomes (Figure 3), is that the asymmetric AChR molecule, with its bulkier extracellular domain, exerts a stronger hindrance to the quenching phenomenon from the exofacial compartment.

Measurement of the fluorescence lifetimes of the probes incorporated into AChR-rich membranes was also carried out using the phase shift and modulation of the exciting signal at three modulation frequencies. The lifetime of *N*-Rho-PE incorporated into the AChR membrane did not vary for modulation frequencies between 6 and 30 MHz, yielding a mean value of 1.4 ± 0.1 ns. Similar invariance was observed with 12-NBD-PC (3.7 ± 0.2 ns).

Since the quenching parameters depend on the lifetime of the excited state of the fluorophore, as well as on the quencher−fluorophore collision rate, nanosecond fluorescence lifetimes of the lipid analogs were measured in the AChR-rich membrane in the presence of the quencher cobaltous ion. In the presence of increasing Co^{2+} concentrations (0−30 mM), the fluorescence lifetime of *N*-Rho-PE in the AChR-rich membrane, as measured by phase modulation, progressively diminished from 1.4 to 0.8 ns. Similar results were observed with 12-NBD-PC (from 3.5 to 1.7 ns).

Had we been dealing with a case of pure collisional quenching, the plots of F_o/F and of τ_o/τ versus cobalt concentration would have overlapped within experimental error. As Figure 4 shows, however, this is not the case: the plot of F_o/F yielded consistently higher values than that of τ_o/τ at different cobalt concentrations. The quenching of the

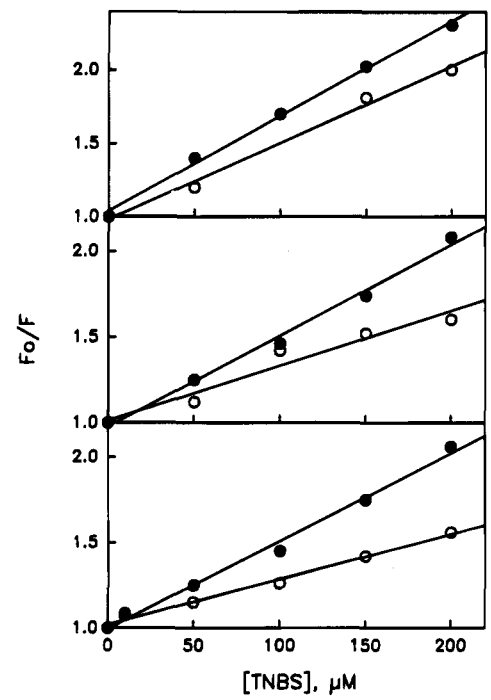

FIGURE 5: Stern−Volmer plot showing (from top to bottom) the quenching by TNBS of the extrinsic fluorescence of *N*-Rho-PE, 12-NBD-PC, and NBD-PE in AChR-rich membranes under nonpermeabilizing (4 °C, ○) or permeabilizing (37 °C, ●) conditions.

fluorescence of NBD-phospholipids and *N*-Rho-PE by cobalt is thus of a mixed type, most likely the sum of a collisional plus a static type of quenching [see, for example, Matko et al. (1992)]. It should be noted that Co^{2+} is paramagnetic (S = 3/2 and 1/2) and has a quenching radius that is greater than its van der Waals radius. This may account for the observation of mixed static and dynamic quenching with this ion. It should be noted that saponin does not significantly increase the fraction of Rho-PE in AChR-rich membrane accessible to quencher, but it does increase its K_{SV}. This is consistent with the hypothesis that a fraction of Rho-PE is partly inaccessible to Co^{2+} quenching by the presence of the protein in native membranes, but not in liposomes reconstituted from lipids only. Such a hypothesis is supported by the data on polarization of fluorescence and lifetime determinations of Rho-PE in AChR-rich membranes. Incidentally, the fact that saponin does not increase f_a provides additional evidence supporting the notion that the percentage of multilayered vesicles in the AChR-rich membrane fraction must be very low: in fact, less than 3%.

The observation that the quenching data in Figure 4 do not approach 1 as the Co^{2+} concentration approaches 0 indicates that we are dealing with a heterogeneous population of fluorophores, as noted by Eftkin and Ghiron (1981). This appears to be the case here, because the adsorption of Co^{2+} to discrete sites at the lipid−water interface allows us to differentiate between NBD-labeled lipids on the basis of their proximity to bound Co^{2+}. The very different K_{SV} constants obtained for Co^{2+} quenching of NBD-labeled lipids in PC liposomes and in PC/PS liposomes (Homan and Eisenberg, 1985) support this view.

The fluorescence of NBD-PC, NBD-PE, and *N*-Rho-PE in AChR membranes was efficiently quenched by TNBS (Figure 5 and Table 3). Under nonpenetrating conditions at

Table 3: Fluorescence Quenching by TNBS of Extrinsic Lipid Probe Fluorescence in AChR-Rich Membranes from *T. marmorata*[a]

	K_{SV} (mM^{-1})		$1/K_{SV}$ (mM)	
probe	4 °C	37 °C	4 °C	37 °C
12-NBD-PC	2.5	5.0	0.40	0.20
N-Rho-PE	5.0	6.0	0.20	0.16
NBD-PE	2.8	5.0	0.35	0.20

[a] Values for the quenching parameters were derived from a Stern–Volmer analysis of the data obtained under penetrating (37 °C) and nonpenetrating (4 °C) conditions.

4 °C, the fraction of fluorophores available for quenching by TNBS was found to be similar to that for Co^{2+} quenching (cf. Figure 3 and Table 2). f_a does not change significantly between 4 and 37 °C, that is, between nonpermeabilized and permeabilized membranes, respectively (Figure 5).

The parameter $1/K_{SV}$ in Table 3 represents the concentration of quencher necessary to reduce the initial fluorescence intensity by half, reflecting the efficiency of the process. Quenching efficiency is lower for both NBD-PC and NBD-PE than for *N*-Rho-labeled lipids incorporated into AChR membranes. It is also apparent from Table 3 that upon membrane permeabilization the fluorophores become more accessible to the quencher. The fraction of labeled lipid (f_a) accessible to TNBS is the same at 4 and 37 °C (cf. Figure 5). These results are consistent with those of Co^{2+} quenching, showing predominant exofacial location of the labeled lipid in AChR membranes. As expected, the K_{SV} values are increased upon passing from 4 to 37 °C, probably owing to the effect of temperature on the diffusional rate of the quencher.

DISCUSSION

The exact topography of the major phospholipid classes in the cholinergic postsynaptic membrane is still poorly understood. This is partly due to the relative lack of appropriate methodologies for providing answers while preserving the integrity of the membrane. In an attempt to tackle this issue, we have developed a spectroscopic approach using fluorescent derivatives of the two major phospholipid classes present in AChR-rich membranes, i.e., PC and PE (cf. Rotstein et al., 1987a).

Consistent with the TNBS labeling of phospholipids in AChR membranes (Bonini de Romanelli et al., 1990), and with the phospholipase C hydrolysis experiments, the fluorescence energy transfer and quenching experiments presented here show that NBD-PC and Rho-PE preferentially partition in the same leaflet of the bilayer. This conclusion essentially stems from the comparison of the fluorescence energy transfer at constant donor concentrations of model lipid vesicles reconstituted from a PC fraction prepared from endogenous lipid extracts of the *Torpedo* AChR-rich membrane or synthetic dioleoyl-PC on the one hand and native AChR-rich membranes on the other, indicating that the two probes reach such high energy transfer efficiencies because they are both located in the *same leaflet* of the bilayer (Figures 1 and 2). The results obtained with AChR-rich native membranes were very similar to those observed with preparations of liposomes containing both fluorophores in the external face of the membrane (Table 2), in agreement with the results of phospholipase C digestion. This series of experiments suggests that the *parental* phospholipids, i.e., PC and PE, are also located predominantly in the exofacial leaflet. Furthermore, the analysis of the efficiency of energy transfer suggests deviations from a homogeneous distribution within this leaflet at the higher molar ratios of acceptor lipid used in liposomes prepared with the endogenous PC fraction obtained from AChR-rich membrane lipid extracts.

In order to establish more precisely the topography of NBD-PC and Rho-PE in AChR membranes, parallel quenching studies with cobaltous and TNBS ions were undertaken. Both ions are efficient water-soluble quenching agents, and both are expected to remain in the aqueous phase. Partial exposure of the fluorophore to the lipid–water interface, rather than penetration of the quencher in the membrane, is likely to be responsible for the relatively high quenching efficiencies observed (Tables 2 and 3), in close agreement with values reported, e.g., for NBD-PE (Homan & Eisenberg, 1985), and with the location of the NBD group of NBD-labeled lipids, i.e., in proximity to the lipid–water interface (Chattopadhyay, 1990).

The lower efficiency of TNBS quenching of NBD-labeled as opposed to *N*-Rho-labeled lipids in AChR membranes, as reflected in the observed K_{SV} values, suggests that the latter fluorophore is more exposed to the lipid–water interface than the NBD group. Similar conclusions were recently reached by Wolf et al. (1992). Furthermore, the location of the NBD group within the phospholipid molecule does not seem to be of decisive relevance for its location in the membrane, in agreement with the studies of Chattopadhyay and London (1987, 1988) on the ionization properties of the NBD group covalently bound to PC or PE. The NBD moiety appears to lie close to the polar head region of either phospholipid. For this to occur, the NBD at the C terminal of lauric acid in 12-NBD-PC has to form a loop, with concomitant rearrangement of the acyl chain. Chattopadhyay and London (1987) ascribed this behavior to the high polarity of N and O atoms in the NBD molecule, forcing the fluorophore toward the aqueous interface. Wolf et al. (1992) place the NBD group at or above the lipid phosphate group, 2.0–3.5 nm from the bilayer center. From the very slight temperature dependence of the quenching by TNBS of Rho-PE emission (see the following), one can hypothesize that this could be due to partial protection of the *N*-Rho-PE molecule by the bulkier extracellular domain of the AChR protein in the membrane, since the polarization of Rho-PE in AChR membranes is much higher than that in pure lipid vesicles, and this strongly suggests a preferential location of this lipid at the AChR–lipid interface. In addition, the lifetime values of NBD-PC in AChR membranes were lower than those reported for this probe in large unilamellar lipid vesicles (8.5 ns; Arvinte et al., 1986), indicating that the microenvironment of NBD in AChR membranes differs from that in a pure lipid bilayer.

The fact that the experimental data indicate partition of the probes between annular and bulk lipid does not affect the calculation of the distance between donor and acceptor molecules in the two different hemilayers because (1) the high protein to lipid ratio in AChR-rich membranes and the concomitant short receptor–receptor distances (cf. Barrantes, 1989) imply that at least 50% of the total lipid is in contact with at least one AChR molecule, and (2) the width of the bilayer, 5–6-fold of the diameter of a phospholipid molecule, determines, on average, 3-4 belts of AChR-associated phospholipid per AChR molecule. These two reasons reduce

the error in calculating the distance to less than 0.2 nm, implying that within the experimental accuracy of our measurements both the assumption that the donor and acceptor molecules partition between annular/nonannular lipid and the case of random distribution of acceptor and donor molecules within a hemilayer of the membrane fit the data equally well.

It is also pertinent to comment on the possibility that our data could be vitiated by two experimental handicaps: (i) transbilayer mobility of the phospholipid probes used within the time scale of our measurements and (ii) occurrence of multilamellar or multivesicular systems in our preparations. With respect to the former possibility, McNamee and McConnell (1973) reported a very high transleaflet mobility of PC using a membrane preparation from a different species, the Gymnotidae *Electrophorus electricus*. In addition to the inherent biological differences between the two preparations, which may be responsible for the unusual values of PC transleaflet mobility, *Electrophorus* vesicle preparations are much less rich in AChR protein than those from Torpedinidae species and subsequently have not been used for this type of study. Measurements of other parameters of lipid mobility (rotational diffusion coefficient, lateral diffusion coefficient) did not show any peculiarity in AChR-rich membranes [see the review by Barrantes 1989)], and PC transbilayer flip-flop usually exhibits half-times of hours [see review in Thompson and Huang, (1978)]. With respect to the occurrence of trapped multilamellar or multivesicular vesicles, our resonance energy transfer experiments strongly argue against this possibility. The closest donor–acceptor distances that fit the experimental data satisfactorily coincide with the width of a membrane bilayer and not with the distribution of (the greater) distances expected for multilamellar systems.

From the combined energy transfer, Co^{2+} and TNBS quenching, and lifetime measurements in native AChR and model lipid systems, we conclude that the Rho- and NBD-labeled PE probes are located at the lipid–aqueous interface, i.e., partly exposed to the aqueous medium. In the case of NBD-labeled lipids, the fluorophore reaccommodates itself close to the preceding interface. The experimental data point to the conclusion that NBD-PC and Rho-PE are located in the outer, exofacial membrane leaflet.

Additional information can be derived from our Co^{2+} quenching studies. When fluorescent probes are located close to the lipid–water interface, the value of the quenching constant K_{SV} is sensitive to the membrane surface potential [Toccane and Teissié (1990) and references therein]. The asymmetric distribution of lipids in AChR-rich membranes as reported here should produce a significant negative surface potential in the outer leaflet, which we calculate to be ca. −15 mV using eqs 2 and 3 (see Materials and Methods). The contribution of the negatively charged NBD-labeled lipid itself is not significant at the concentrations used (Homan & Eisenberg, 1985). The lower value of K_{SV} in AChR-rich membrane, as compared to that in liposomes, is likely to be related to an asymmetric distribution of negatively charged lipids, for a surface potential of −15 mV should lead to the observed ΔpK_{SV} of −0.6. In addition, the effects of Co^{2+} on phase and modulation lifetime data of NBD-PC and *N*-Rho-PE in AChR-rich membranes strongly suggest the contribution of static quenching as well, most likely owing to the binding of Co^{2+} to sites close to the lipid–water interface [see, for example, Homan and Eisenberg (1985)] and to the AChR protein. The binding, however, should exert only a minor effect on the ΔpK_{SV} of Co^{2+} quenching. The probability of energy transfer would be increased by the adsorption of Co^{2+} to discrete sites at the lipid–water interface, close to the fluorescent lipid donors. During the lifetime of the donor, the positions of the acceptors in these sites can be looked upon as static [see, for example, Wolber and Hudson (1979); Gutiérrez-Merino, 1981a,b, 1987; Yguerabide, 1994]. As discussed earlier, the location of the NBD group of NBD-labeled lipids is close to the lipid–water interface (Chattopadhyay, 1990), and Co^{2+} adsorption to the lipid–water interface of PC and PC/PS liposomes can be described by the Gouy–Chapman equations for the diffuse double-layer theory (Homan & Eisenberg, 1985).

The difference between the K_{SV} values in liposomes prepared with PC extracted from AChR-rich membranes and those made exclusively of synthetic PC ($\Delta pK_{SV} < 0.3$) merits a brief comment. Because the lifetimes of the fluorescent lipids in AChR-rich membranes show clear indications of environmental microheterogeneity around the probe, it is likely that the fluorescent derivatives partition between the two known lipid compartments—bulk and annular lipids [see Barrantes (1989, 1993)]—in these membranes, and as a result the average location of the probe with respect to the lipid–water interface is altered. In conclusion, the ΔpK_{SV} values are indicative of an inherent asymmetry of the AChR-rich membrane constituents and provide a relative indication of the magnitude of the charge distribution across the two leaflets of the bilayer.

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