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Lipid Environment of Acetylcholine Receptor from *Torpedo californica*†

J. M. Gonzalez-Ros, M. Llanillo, A. Paraschos, and M. Martinez-Carrion*

ABSTRACT: The lipid matrix of both acetylcholine receptor (AcChR)-rich and AcChR-poor membranes from *Torpedo californica* electroplax has been chemically characterized. AcChR-rich membranes contained higher levels of free cholesterol and slightly higher proportions of polyunsaturated ethanolamine phosphoglycerides. Major fatty acid components in the total lipid extracts from either membrane fraction were 24% palmitic, 20% docosahexaenoic, and 20% oleic acids. The fatty acid composition of individual lipid components from AcChR-rich and AcChR-poor membrane fractions was very similar. Native membranes and lipid vesicles, obtained by detergent dialysis from octyl glucoside solubilized lipid extracts, were used for the determination of the temperature-dependent rotational relaxation time of two fluorophores: diphenylhexatriene (DPH) and its trimethylammonium derivative (TMA-DPH). In both the DPH and the TMA-DPH domains, vesicles assembled with AcChR-rich membrane lipid exhibited a higher degree of rigidity than vesicles composed of AcChR-poor membrane lipid. The differences can be partially explained by their different cholesterol levels. As judged by Arrhenius plots, lipid phase separations or transitions were absent within the temperature range used in every native lipid system studied. In all cases, the presence of protein apparently induced more restraint on the rotational motion of the fluorophores. For example, higher rotational relaxation time values were obtained in AcChR-rich membranes than in corresponding plain lipid vesicles. This increase was less apparent with AcChR-poor membranes. Titration of the fluorescent pH probe 4-heptadecyl-7-hydroxycoumarin indicated that the apparent pK of the probe was influenced by the nature of the phospholipids and by the presence of protein. The pK value of coumarin was lower in vesicles formed with AcChR-rich membrane lipid (9.05) than in the intact native AcChR-rich membranes (9.25).

The nicotinic acetylcholine receptor (AcChR)[1] is an integral membrane glycoprotein mainly located at the neuromuscular synaptic junction and in the electric organ of certain elasmobranchs. Binding of specific agonists (acetylcholine) elicits translocation of ions which initiate postsynaptic depolarization. Purified AcChR as isolated from *Torpedo californica* has a molecular weight of 270 000 (Martinez-Carrion et al., 1975) and is composed of four different polypeptide chains (M_r 40 000, 50 000, 60 000, and 65 000) in an apparent 2:1:1:1 stoichiometry (Reynolds & Karlin, 1978; A. Paraschos, J. M. Gonzalez-Ros, and M. Martinez-Carrion, unpublished results).

The 40 000 molecular weight subunit is known to bear the binding site(s) for agonists, antagonists, and α-neurotoxins (Weill et al., 1974; Hsu & Raftery, 1979; Witzemann & Raftery, 1977, 1978; Lyddiatt et al., 1979; Damle et al., 1978) while the 50 000 and 60 000 molecular weight subunits appear to have large domains exposed to the membrane lipid environment (Sator et al., 1979; Gonzalez-Ros et al., 1980a). Few details, however, are available on the molecular mechanisms involved either in the formation of the "ion channel" initiating

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[1] Abbreviations: AcChR, acetylcholine receptor; OG, octyl β-D-glucopyranoside; α-Bgt, α-bungarotoxin; EP, ethanolamine phosphoglyceride; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; CARD, cardiolipin; PA, phosphatidic acid; SPH, sphingomyelin; l-PC, lysophosphatidylcholine; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

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postsynaptic membrane depolarization or in the reversible transitions between "sensitized–desensitized" states of the membrane-bound AcChR. Accordingly, the possible role of membrane lipid and lipid–protein interactions on the mechanisms of AcChR functionality has yet to receive more detailed consideration than currently available (Popot et al., 1978; Schiebler & Hucho, 1978), especially with regard to composition and physical properties. Additionally, little is known concerning the possible association of membrane-bound AcChR to specific lipid patches, as well as the existence and nature of "boundary" lipids (Marsh & Barrantes, 1978; Andreasen & McNamee, 1980).

As part of a systematic study of the lipid–protein interactions involved in AcChR functionality, we describe in detail the lipid composition and some physical properties of different membrane fractions isolated from *Torpedo californica* electroplax. Comparisons between the different membrane fractions are performed in order to facilitate the understanding of features common to the whole electroplax tissue while emphasizing those specific for the AcChR-rich membrane fraction.

Materials and Methods

Isolation of Electroplax Membranes. *Torpedo californica* electroplax, excised and then frozen in liquid nitrogen, was purchased at Pacific Biomarines Supply Co. (Venice, CA) and stored at −70 °C until needed. Prior to use, the tissue was thawed by immersion in an ice-cold beaker containing Ca^{2+}-free Ringer solution (10 mM Tris-HCl, pH 7.4, 262 mM NaCl, 5 mM KCl, 0.1 mM PMSF, and 0.02% NaN_3), and larger pieces of connective tissue were discarded. The electric tissue was minced and homogenized (1:1 w/v) in Ca^{2+}-free Ringer solution using a Sorvall Omnimixer homogenizer at full speed (3 × 2 min). The homogenate was centrifuged at 3500*g* for 15 min, and the supernatant was filtered through eight layers of cheesecloth. The filtered solution was centrifuged at 100000*g* for 1 h and the pellet resuspended in Ringer solution and recentrifuged. The final pellet, referred to as crude or unfractionated electroplax membranes, was resuspended at 6–7 mg of protein/mL in a small volume of Ringer solution containing 25% (w/v) sucrose, layered on the top of a continuous linear sucrose density gradient [25–60% (w/v) sucrose in Ringer solution] and centrifuged in a SW-27 Beckman rotor at 24000 rpm for 2.5 h. Protein concentrations were measured by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Binding of α-Bgt to acetylcholine receptor was determined by using a DEAE-cellulose filter disk assay procedure (Schmidt & Raftery, 1973) where either [^{125}I]-α-Bgt (New England Nuclear) purified by chromatography on DEAE-Sephadex or methylated [^{14}C]-α-Bgt (Calvo-Fernandez & Martinez-Carrion, 1981) was used. Acetylcholinesterase activity was determined by the colorimetric assay of Ellman et al. (1961).

Lipid Extraction, Fractionation, and Characterization. Total lipid from unfractionated or fractionated electroplax membranes was extracted by the procedure of Bligh & Dyer (1959). Lipid extracts were washed with a 0.73% NaCl solution and evaporated to complete dryness. Dried lipid extracts were taken up to approximately 25 mg of lipids/mL in chloroform/methanol (2:1 v/v) and stored under argon at −30 °C until used for further analysis (between 1 and 7 days). Handling of lipid extracts was always done under argon, and all organic solvents were of high quality and glass distilled from Burdick & Jackson Laboratories (Muskegon, MI). Total, free, and esterified cholesterol, total sugars, and phosphorus content were determined simultaneously in the lipid extracts to avoid errors from evaporation of the chloroform/methanol solutions. Cholesterol determinations were done by essentially the Zlatkis et al. (1953) and Courchaine et al. (1959) procedures. Total sugar moieties of lipid extracts were evaluated as basically described by Radin et al. (1956), except that an aqueous phenol solution (Dubois et al., 1956) was used in place of anthrone to estimate the furfural derivatives of sugar moieties upon treatment with 3 N H_2SO_4.

Lipid phosphorus was determined by the micromethod described in Bartlett (1959) except that the Fiske and Subba-Row reagent was prepared by dissolving 62.5 mg of 4-amino-3-naphthol-1-sulfonic acid and 5 g of $Na_2S_2O_5$ up to 25 mL with distilled water.

Neutral lipid classes were fractionated by thin-layer chromatography (TLC) on activated (1 h, 110 °C) 0.25-mm layers of silica gel G (Redi-Plate, Analtech) using hexane/ethyl ether/acetic acid/methanol (60:40:1:1 v/v) as the developing solvent. After development, the plates were sprayed with a 0.05% Rhodamine solution in methanol, and lipid bands were visualized under UV light.

Polar lipids were fractionated by two-dimensional TLC on activated 0.2-mm layers of linear high performance 10 × 10 cm plates (LHP-K, Whatman). Aliquots containing 100–150 μg of total lipid were developed twice (developed, dried under a stream of argon, and then developed again) using chloroform/methanol/concentrated ammonia (65:24:4 v/v). Plates developed twice in the first dimension were dried for 15 min under a stream of argon and 30 min under vacuum and then developed with chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5 v/v) in the second dimension. It is crucial that all traces of NH_4OH be removed prior to chromatography on the second dimension. Lipid spots were visualized by charring with 10 N H_2SO_4 (for samples for phosphorus determinations) or by spraying with phospray (Supelco) (for identification of phosphorus containing lipids) or with a solution of 1′,7′-dichlorofluorescein (Supelco) (for samples for fatty acid analysis). Quantitation of the different diacyl phospholipids vs. the corresponding alk-1-enyl (plasmalogens) derivatives was achieved through a separation–reaction–separation two-dimensional TLC procedure similar to the one described by Owens (1966).

For preparative purposes, total phospholipids from lipid extracts were separated from neutral lipid by TLC on 0.25-mm layers of silica gel G (Redi-Plate, Analtech) using hexane/ethyl ether/acetic acid/methanol (60:40:1:1 v/v) as the solvent system. Upon visualization with 0.05% Rhodamine solution in methanol under UV light, the phospholipid band was scrapped off the plate and eluted from the adsorbent as previously described (Gonzalez-Ros & Ribera, 1980).

Transmethylation of total or fractionated lipid classes was performed with a sodium methoxide/methanol reagent (methanolic base, Supelco). Fatty acid methyl esters were extracted on a water–petroleum ether system, taken to dryness under argon in small conical centrifuge tubes, and finally dissolved in 20-μL aliquots of isooctane for analysis by gas–liquid chromatography (GLC).

GLC analyses were performed on a Varian 3700 gas chromatograph equipped with a dual flame ionization detector and with 6-ft glass columns (2-mm internal diameter). Methyl pentadecanoate and cholesterol were used as internal standards to validate the above described colorimetric determinations on individual phospholipids and cholesterol, respectively.

Preparation of Lipid Vesicles. Total lipid extracts from the different membrane fractions were used to produce large lipid vesicles by a detergent dialysis method (Petri & Wagner, 1979; Gonzalez-Ros et al., 1980b). Enough lipids to provide 1–2

mL of a suspension with a concentration of 1 mM in lipid phosphorus were solubilized with octyl β-D-glucopyranoside (OG) in vesicle buffer (10 mM Tris, pH 7.4, 262 mM NaCl, 5 mM KCl, 1 mM EDTA, and 0.02% NaN_3) saturated with argon. The detergent/lipid mixtures were extensively dialyzed in the cold under argon against vesicle buffer (4 × 1 L changes within 48 h), and large lipid vesicles were formed as the detergent was removed. Complete removal of OG during the dialysis was determined by using octyl β-D-[U-^{14}C]glucopyranoside (New England Nuclear) as radioactive tracer. Upon dialysis, phosphorus determination was repeated on the lipid vesicles and the final lipid phosphorus concentration adjusted to 50 μM unless otherwise indicated. The adjustment of the lipid phosphorus concentration on the different lipid vesicles being compared was found to be critical in securing constant "dilute" polarization values which otherwise would be dependent upon the concentration and/or light scattering of the samples (Lentz et al., 1979). Lipid vesicles for fluorescence measurements were used within a maximum of 48 h following detergent dialysis. When the phospholipid/cholesterol ratio in the different lipid vesicles was to be altered, known amounts of dry cholesterol and/or total native phospholipids were either mixed together or added to the lipid extracts prior to OG solubilization.

Fluorescence. Fluorescence spectra, polarization, and lifetime measurements were performed with an automated scanning SLM 4800 series spectrofluorometer (SLM, Champaign, IL) equipped with a Hewlett-Packard 9815 A and 9862 A calculator and plotter, respectively. Temperature was controlled by a Haake thermostated water bath and measured inside the fluorescent cuvettes with a YSI probe (Yellow Springs Instruments, Yellow Springs, OH). Polarizers in the excitation and both emission beams were used to measure fluorescence depolarization. DPH or TMA-DPH (1 mM; Molecular Probes) dissolved in UV grade tetrahydrofuran or methanol, respectively, was added to the lipid vesicles or native membranes at a ratio of 1 molecule of DPH or TMA-DPH for every 1000 phospholipid molecules, and after 2-h incubation at 40 °C the probes were excited at 365 nm with slit widths set at 4 nm. Altering the fluorophore/phospholipid ratio from 1:250 to 1:1000 did not produce any alteration on the polarization or lifetime values. Corning 3-73 filters were used in both emission beams to eliminate scattered light. A complete discussion on the use of DPH as a probe for the hydrophobic region of the lipid bilayer is reported in Shinitzky & Barenholz (1974).

Fluorescence lifetimes were measured by the cross-correlation phase method described by Spencer & Weber (1969). Lifetime measurements were made with the excitation light modulated at 18 MHz. The average of the phase and modulation values relative to a glycogen scattering solution was used for all calculations.

Rotational relaxation times ($\bar{\rho}$) for DPH and TMA-DPH were calculated by using the Perrin equation

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\bar{\rho}}\right)$$

where P is the polarization, τ is the lifetime of the excited state, and P_0 is the polarization in the absence of rotational motion. P_0 has been reported to be 0.460 for DPH (Shinitzky & Inbar, 1974). Moreover, TMA-DPH and DPH have been shown to have identical limiting polarization values (Prendergast et al., 1981). The validity of the above equation for a nonspherical fluorophore like DPH has been previously discussed (Shinitzky et al., 1971).

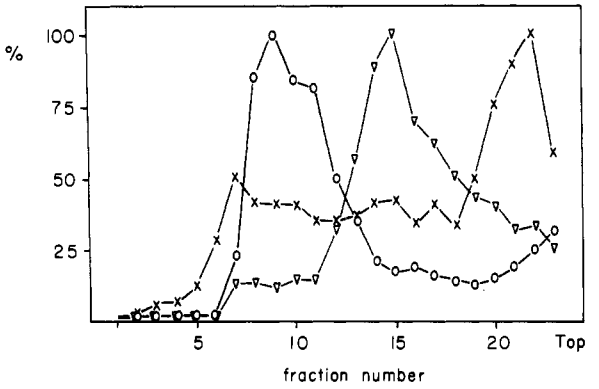

FIGURE 1: Continuous sucrose density gradient [25–60% (w/v) sucrose] centrifugation of crude *Torpedo* electroplax membranes. Fractions were collected from the bottom of the gradients, and specific α-Bgt binding (▽), acetylcholinesterase activity (×), and total protein content (○) were determined. In all cases, 100% relative values were assigned to the highest values from the fractionated samples.

4-Heptadecyl-7-hydroxycoumarin dissolved in tetrahydrofuran was added to the vesicle or membrane samples at a fluorophore to phospholipid molar ratio of 1:200 and incubated for 1 h at 40 °C. Incorporation of the probe into the lipid matrix was followed by monitoring the enhancement of the fluorescence intensity or the changes in fluorescence polarization. Fluorescence excitation spectra were recorded either at 450-nm fixed emission wavelength or by using a Corning 3-72 filter in the emission beam with all slit widths at 4 nm. Samples containing the coumarin probe were titrated starting at pH 7.4 by adding small aliquots of 0.1 M NaOH or 0.1 M HCl. Measurements of pH were done on the same samples used to obtain the fluorescence excitation spectra. The degree of ionization of the fluorescence probe was estimated either by the ratio of the areas of the peaks at 342 and 383 nm for the un-ionized and ionized molecule, respectively, or by the ratio of both fluorescence intensity maxima. The apparent p*K*s determined by either method were essentially identical. The p*K* values were estimated as the pH at the inflection point in the titration curve.

Results

(*1*) *Membrane Isolation.* A typical profile of a sucrose density gradient fractionation of crude electroplax membranes is shown in Figure 1. A major α-Bgt binding peak separated from bulk membrane protein and was observed toward lower density regions of the gradient. Additionally, the α-Bgt binding enriched peak was well separated from the acetylcholinesterase specific activity peak. Several fractions were pooled in order to obtain (a) the AcChR-rich membranes which exhibited the highest specific activity for α-Bgt binding and (b) the more dense AcChR-poor membranes which were enriched in bulk membrane protein. In general, we refer to fractions 7–9 (Figure 1) as AcChR-poor membranes and fractions 14–16 as AcChR-rich membranes. Unfractionated membranes contain these and other membrane particles as described under Materials and Methods. Characterization of the pooled membrane samples revealed a significant difference between AcChR-rich and AcChR-poor membranes with respect to their specific activities for α-Bgt binding (approximately 1.5 and 0.1 nmol of α-Bgt bound/mg of protein, respectively). These two membrane samples as well as an aliquot from the unfractionated crude electroplax membranes were used for subsequent experiments.

(*2*) *Lipid Composition of Electroplax Membrane Fractions.* Lipid to protein ratios for the different electroplax membrane

Table I: Total Lipid Contents of Electroplax Membranes[a]

	lipid/protein[b] (w/w)	phospholipid/ cholesterol[c] (molar ratio)
unfractionated membranes	0.57 ± 0.28 (7)	1.41 ± 0.27 (7)
AcChR-rich membranes	0.43 ± 0.12 (6)	1.10 ± 0.27 (8)
AcChR-poor membranes	0.30 ± 0.07 (6)	1.77 ± 0.16 (6)

[a] Values are mean ± standard deviation, followed in parentheses by the number of membrane preparations used for the determination. [b] Total lipid and proteins were estimated gravimetrically and by Lowry's procedure, respectively. [c] Total phosphorus and cholesterol were determined by colorimetric procedures (see Materials and Methods).

fractions were determined (Table I). All fractions analyzed appeared to have a much lower lipid content than topographically related membranes such as electroplax synaptic vesicles (Deutsch & Kelly, 1981). Lipid to protein ratios obtained for the AcChR-rich membranes agree closely with previously published results from Popot et al. (1978) with *Torpedo marmorata*. Lipid to protein ratios for the AcChR-rich membranes were always higher and had a higher cholesterol content than AcChR-poor membranes (Table I). The ratio of phospholipid to cholesterol of the AcChR-rich membranes is similar to that of lipid-rich synaptic vesicles (Deutsch & Kelly, 1981).

The neutral lipid classes of electroplax membranes were composed exclusively of an unesterified sterol fraction which upon GLC analysis (data not shown) indicates only cholesterol as sole component. No triglycerides or other neutral lipids were detected. Sugar-containing lipids, identified on TLC as a mixture of cerebrosides, sulfatides, and gangliosides, account for less than 2% of the total lipid extracts on a molar basis. These calculations were obtained assuming one sugar residue per glycolipid, with 100% being the summation of the molar concentration of sugar, lipid phosphate, and cholesterol.

The phospholipid composition of the different membrane types are summarized in Table II. The recovery of phosphate from the TLC plates was within the 90–100% range; therefore, the possibility of undetected phospholipids in the lipid extracts is unlikely. In all cases, PC, PS, and EP were the major phospholipid components. Moreover, all EP is composed of approximately 60% of $HgCl_2$-stable diacyl derivatives (PE) and 30% of $HgCl_2$-unstable molecules suspected of being ethanolamine plasmalogens. AcChR-poor membranes exhibited approximately equal levels of PC and significantly more PS than the AcChR-rich membranes. The lipid extracts from AcChR-rich membranes contained significantly more EP than either unfractionated or AcChR-poor membrane lipid extracts. Minor phospholipid components were CARD, PA, SPH, and l-PC. Phosphatidylinositol was not detected.

Total fatty acid composition of lipids from unfractionated, AcChR-rich, and AcChR-poor membranes reveals that palmitic (16:0), stearic (18:0), oleic (18:1), arachidonic (20:4), and docosahexaenoic (22:6) acids account for more than 80% of the total fatty acids, and no significant differences could be detected in the various membrane fractions.

In contrast, when the fatty acid composition of the major phospholipid classes in AcChR-rich and AcChR-poor membranes[2] was determined (Table III), the different phospholipid

[2] The fatty acid composition of the major phospholipid classes for the crude unfractionated membranes is quite similar to those shown in Table III and is available from the authors upon request.

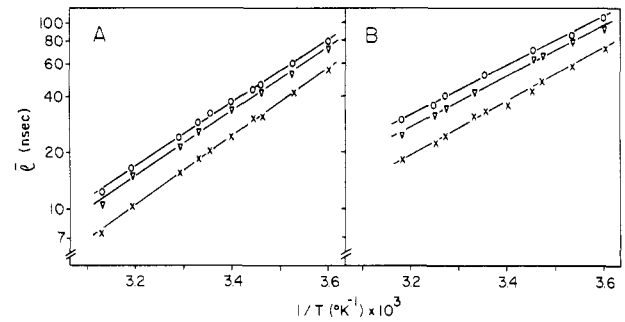

FIGURE 2: Representative temperature dependence of the rotational relaxation time ($\bar{\rho}$) of DPH (A) and TMA-DPH (B) in plain lipid vesicles. Vesicles were produced by the detergent dialysis method (see Materials and Methods) with total lipids from unfractionated (○), AcChR-rich (▽), and AcChR-poor (×) membranes from *Torpedo* electroplax. Fluorescence polarization and lifetime were measured at the indicated temperatures, and the rotational relaxation time values were calculated by the Perrin equation as indicated under Materials and Methods.

classes within the same membrane preparation exhibited very different compositional patterns. Palmitic acid (16:0) was primarily associated with PC while EP, the major phospholipid class in AcChR-rich membranes, contained docosahexaenoic acid (22:6) as the more abundant fatty acid component. Docosahexaenoic acid (22:6) was also abundant in PS, although the major fatty acid was stearic acid (18:0). Oleic acid (18:1) was the major component in CARD. An analysis of the average degree of unsaturation and the average chain length for the fatty acids from the different phospholipids (Table III) reveals that the highly unsaturated classes (EP) had more than six double bonds per phospholipid molecule and an average chain length of 19.6 carbons. However, a lower degree of unsaturation (two double bonds per molecule) appears to be associated with shorter average chain lengths particularly in PC and CARD. A comparison of the AcChR-rich and AcChR-poor fractions shows that while the EP populations seemed identical, PC molecules appeared shorter and less unsaturated in AcChR-rich fractions. Conversely, PS contained slightly longer and more unsaturated acyl residues while CARD and PE exhibited fatty acyl chains with identical lengths, but slightly more unsaturation than in AcChR-poor membranes. Other minor components which are not shown in Table III, such as PA or the mixture SPH + 1-PC, were characterized by a relatively low degree of unsaturation of approximately 1.2 double bonds per fatty acid residue in all the membrane fractions.

Quantitation by GLC analysis correlated well with the phospholipid abundances obtained by phosphorus determinations (Table II). From the fatty acid composition of the individual phospholipids and the relative abundance of each phospholipid within the total lipid population, the total recovery of fatty acids was calculated (Table III). Similarity between the above values and the experimental total fatty acid composition indicate that all the lipid components were accounted for.

(3) *Fluorescence Studies.* (*a*) *Rotational Relaxation Time of DPH and TMA-DPH.* Arrhenius plots were diagramed (Figure 2) to depict the temperature dependence of the rotational relaxation time ($\bar{\rho}$) for DPH (Figure 2A) and TMA-DPH (Figure 2B) in lipid vesicles prepared from unfractionated, AcChR-rich, and AcChR-poor membrane lipid extracts. Three sources of information were provided by these plots: (i) the linearity of the Arrhenius plots was shown; (ii) the higher absolute values for the rotational relaxation time of TMA-DPH compared to DPH suggestive of a more rigid nature of

Table II: Phospholipid Composition of Electroplax Membrane Fractions (Molar Percentages)

	unfractionated membranes		AcChR-rich membranes		AcChR-poor membranes	
	first procedure[a]	second procedure[b]	first procedure	second procedure	first procedure	second procedure
phosphatidylcholine	39.1	40.8	38.4	37.1	40.1	36.7
ethanolamine phosphoglyceride	35.6	33.9	42.2	44.0	32.1	32.8
phosphatidylethanolamine	26.1	ND	35.8	ND	25.6	ND
phosphatidylserine	13.8	19.8	10.9	10.9	18.3	19.0
sphingomyelin + lysophosphatidylcholine	8.6	1.9	2.3	3.2	6.4	7.5
cardiolipin	2.3	2.0	3.3	2.5	2.2	2.1
phosphatidic acid	0.5	1.6	2.9	2.2	0.8	2.0

[a]Phospholipid composition based on duplicate determinations of phosphorus contents by Bartlett's procedure. [b]Phospholipid composition based on single determinations by GLC using an internal standard. ND, not determined.

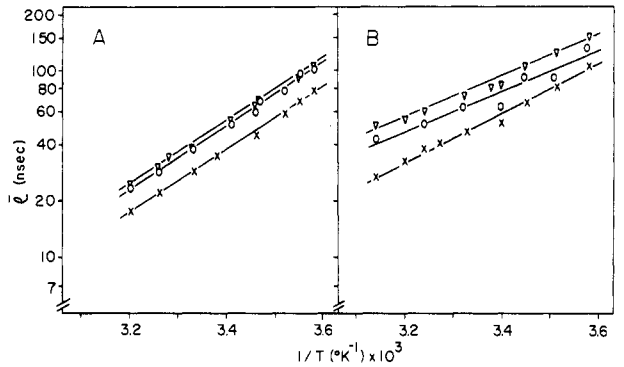

FIGURE 3: Representative temperature dependence of the rotational relaxation time ($\bar{\rho}$) of DPH (A) and TMA-DPH (B) in unfractionated (O), AcChR-rich (∇), and AcChR-poor (×) native membranes from *Torpedo* electroplax. Fluorescence measurements are depicted as in Figure 2.

the surface membrane domain probed by TMA-DPH were shown; and (iii) with both fluorophores the AcChR-poor membrane lipids provided a more fluid matrix (lower rotational relaxation time values) than AcChR-rich membrane lipids, the latter resembling that of lipid vesicles from crude unfractionated membranes.

Similar studies were conducted on unfractionated, AcChR-rich, and AcChR-poor intact *native* membranes to study the effect of proteins present within the hydrophobic matrix (Figure 3). Results revealed an approximate 1.5–2-fold increase in rotational relaxation time values for all samples in the domains probed by either DPH or TMA-DPH. In other words, the presence of membrane protein produced a higher degree of restriction which hindered the rotation of both fluorophores at their respective membrane domains. The most striking difference in behavior between native membranes and lipid vesicles was exhibited by the native AcChR-rich fraction which provided the more ordered environment, especially at the TMA-DPH domain. The AcChR-poor membranes provided the more fluid matrix for both DPH and TMA-DPH. As an additional control, intact native membranes were solubilized with OG, reconstituted by detergent dialysis, and utilized in the fluorescence studies. These reconstituted vesicles which contained native protein exhibited similar lipid fluidity measurements as observed with intact native membranes.

(*b*) *Rotational Relaxation Time of DPH in Vesicles with Varying Phospholipid to Cholesterol Ratio.* For correlation of the value of the rotational relaxation time with a given lipid composition within the lipid matrix, vesicles were prepared with mixtures of synthetic cholesterol and native phospholipids isolated from membrane lipid extracts. The phospholipid to cholesterol molar ratios in these vesicles ranged from 0.5 to

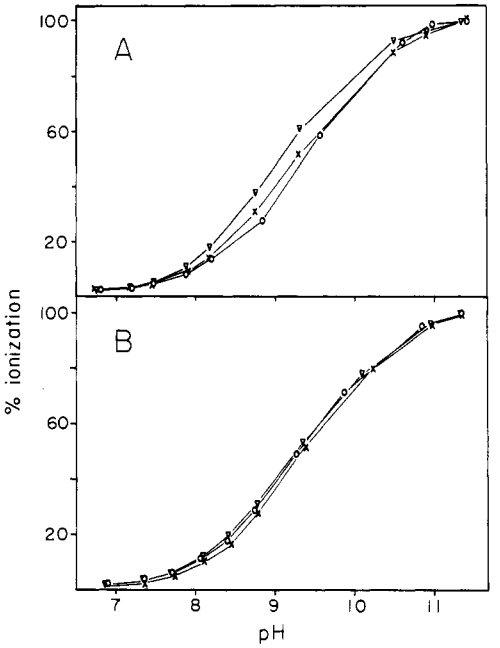

FIGURE 4: Titration of 4-heptadecyl-7-hydroxycoumarin incorporated into plain lipid vesicles (A) and native membranes (B) from unfractionated (O), AcChR-rich (∇), and AcChR-poor (×) membrane fractions from electroplax. Fluorescence measurements are presented as discussed under Materials and Methods. The degree of ionization was calculated from the ratio of the fluorescence intensities at the excitation maxima of the ionized (383 nm) to that of the un-ionized (342 nm) molecule at the indicated pHs.

3.0, spanning in excess the experimental range exhibited by the different lipid extracts. Furthermore, we also analyzed vesicles composed exclusively of native phospholipids in the absence of cholesterol. In all cases, the increase in rotational relaxation time paralleled the decrease in the phospholipid/cholesterol ratio (data not shown). The presence of cholesterol, in addition to protein, produced a higher degree of order within the lipid matrix. When the phospholipid to cholesterol ratios in these vesicles approached the values found in the native lipid extracts, the rotational relaxation time values obtained were very similar to those measured in vesicles prepared with whole lipid extracts.

(*c*) *Titration of 4-Heptadecyl-7-hydroxycoumarin in Lipid Vesicles and Native Membranes.* The fluorophore 4-heptadecyl-7-hydroxycoumarin was used as a probe with plain lipid vesicles and native membranes from unfractionated, AcChR-rich, and AcChR-poor membrane fractions. The hydrophilic nature of the hydroxycoumarin polar head group presumably dictates the position of the probe near the membrane surface aligned with the phospholipid polar head groups with the

Table III: Fatty Acid Composition of Phospholipid Classes (Weight Percent)

fatty acid	phosphatidylcholine		ethanolamine phosphoglyceride		phosphatidyl-ethanolamine		phosphatidylserine		cardiolipin		total recovery[a] of fatty acids	
	AcChR-rich fraction	AcChR-poor fraction	AcChR-rich fraction	AcChR-poor fraction	AcChR-rich fraction	AcChR-poor fraction	AcChR-rich fraction	AcChR-poor fraction	AcChR-rich fraction	AcChR-poor fraction	AcChR-rich fraction	AcChR-poor fraction
14:0	1.0	1.0	TR	Nd[b]	Nd	Nd	TR	TR	Nd	Nd	TR	TR
16:0	51.5	45.0	6.0	6.5	6.9	8.2	7.1	7.4	10.4	11.4	22.8	20.3
16:1	8.4	5.2	3.7	3.5	2.9	3.2	2.2	2.6	5.3	5.1	5.1	3.7
18:0	2.4	2.6	13.9	14.9	17.5	17.4	28.5	30.7	4.6	2.5	10.2	11.7
18:1	18.5	20.9	19.0	18.3	17.3	19.9	15.6	16.6	41.9	41.4	18.0	17.7
18:2	1.6	2.2	1.8	2.0	1.8	2.6	2.3	2.4	10.1	9.2	1.9	2.1
20:1	1.6	2.8	2.3	2.1	2.5	2.5	2.2	2.2	3.9	3.7	TR	2.2
20:2($\omega-6$)	Nd	Nd	Nd	Nd	TR	Nd	TR	TR	3.1	2.5	Nd	Nd
20:4	2.2	3.4	10.5	10.7	13.2	10.3	6.5	8.5	TR	TR	6.1	6.4
20:5	2.8	4.2	3.8	4.6	6.4	5.5	2.8	2.6	1.1	0.8	3.1	3.6
21:5($\omega-1$)	TR	TR	1.3	1.1	1.1	1.0	1.2	1.0	TR	TR	0.7	0.5
22:1	0.9	1.0	0.9	0.9	1.0	2.6	3.1	2.2	12.8	21.8	1.3	1.5
22:5($\omega-6$)	TR	TR	0.5	0.5	TR	TR	0.8	0.6	TR	TR	TR	TR
22:5($\omega-3$)	0.9	1.2	5.1	4.3	4.3	4.2	5.0	3.8	1.5	TR	3.1	2.6
22:6($\omega-3$)	8.2	11.3	31.0	30.4	25.0	22.5	22.6	19.4	5.4	1.6	19.3	18.1
av degree of unsaturation	1.1	1.4	3.1	3.1	2.9	2.6	2.4	2.2	1.4	1.1		
av chain length	17.3	17.7	19.7	19.6	19.5	19.4	19.3	18.9	18.6	18.7		

[a] Recovery of fatty acids is calculated on the basis of molar relative abundances and fatty acid composition of individual phospholipids through the equation $a_{i,\text{total}} = \sum_j a_{ij} P_j / 100$, where $a_{i,\text{total}}$ represents the recovery of the different i fatty acids in the total lipid fractions. a_{ij} is the weight percentage of each i fatty acid in each j phospholipid, and P_j is the molar percentage of each j phospholipid within the total phospholipid population. [b] Nd, not detected; TR, trace.

heptadecyl moiety anchored in the hydrophobic domain of the bilayer (Petri et al., 1981). Since the excitation spectrum of the fluorophore is pH sensitive (Figure 4), the fluorophore can be used to probe the ionic environment near the membrane surface by measuring its apparent pK from its fluorescence excitation spectrum (Fromherz, 1973). Titration of the coumarin probe in lipid vesicles revealed apparent pK values of 9.35, 9.05, and 9.25 when prepared with unfractionated, AcChR-rich, and AcChR-poor membrane lipid extracts, respectively. However, the presence of proteins in the intact native membranes appeared to normalize the pK of the coumarin probe (9.25, 9.25, and 9.30 for crude, AcChR-rich, and AcChR-poor membranes, respectively).

Discussion

In a recent paper by Loh & Law (1980), the role of membrane lipids in receptor mechanisms was discussed on the basis of protein–lipid interactions within the fluid mosaic membrane model. Three different roles were proposed: (i) the lipid alone or in combination with a membrane protein serves as the ligand binding site; (ii) the lipid environment surrounding the receptor molecule affects the conformation of the receptor protein and thus the ligand affinity for the receptor; and (iii) the physical state of the lipid domain where the receptor is located regulates the ability of the receptor molecule to undergo motion, thus, modulating receptor–effector interactions. In the case of AcChR where the receptor–effector systems are contained within the protein complex, Loh and Law concluded that there might be a possible involvement of lipids in AcChR action, although the controversial nature of results from different groups and the lack of evidence makes it difficult to reach definitive conclusions. The possible role of lipids acting as ligand binding sites seems unlikely since α-Bgt and other cholinergic ligand binding sites have been positively identified on the 40 000 molecular weight subunit of the AcChR protein. On the other hand, the purified AcChR protein exhibits ligand dissociation constants which differ by an order of magnitude from those obtained from membrane-bound AcChR (Raftery et al., 1974, 1975; Farach et al., 1982). Affinity changes for cholinergic ligands are also known to be associated with sensitization–desensitization transitions. Additionally, slight perturbations of the lipid matrix as described by many authors using a variety of procedures (Weiland et al., 1977; Young et al., 1978; Hanley, 1978; Andreasen & McNamee, 1977, 1980; Andreasen et al., 1979) seem to result in the loss of the ability of the membrane-bound AcChR to undergo sensitization–desensitization-like transitions, judged by an α-Bgt time-dependent assay, or to promote cation fluxes. Hence, the role of lipids in maintaining or even directing defined physical states which modulate the capacity of the AcChR to undergo conformational changes which may control the associated ion channel necessitates further investigation.

It is not surprising that results obtained in attempts at reconstituting the AcChR ion translocating activity from purified protein in the presence of tightly bound detergent or from nonnative lipid mixtures have led to preparations of membrane vesicles which vary in their degree of efficiency for agonist-induced monovalent ion translocation. In our reconstituted model system we use (i) lipid-free AcChR protein isolated from solubilized extracts where the detergent was loosely bound to the protein and easily dialyzable from the detergent/lipid/protein reconstituting mixtures (Gonzalez-Ros et al., 1981) and (ii) the native electroplax membrane lipids to provide the proper lipid medium for the reconstituted AcChR protein (Gonzalez-Ros et al., 1980b). These results have encouraged us to study the chemical composition and

physical properties of the electroplax lipid to facilitate exploration of specific lipid features associated to the presence of AcChR within the electroplax membrane system.

The crude native membranes and fractionated membranes exhibited AcChR specific activities which were slightly variable between preparations. Variability was quite evident in the lipid/protein ratios (Table I) determined for the crude membranes. To reduce potential variability, we have used only AcChR-rich membranes with specific activities for α-Bgt binding 8–10-fold higher (approximately 1.5 nmol of α-Bgt binding sites/mg of protein) than those of the AcChR-poor membranes. Real differences observed between total lipid extracts from AcChR-rich and AcChR-poor membranes were the higher amounts of lipid and the higher proportion of cholesterol to phospholipid in the AcChR-rich membranes. Nonesterified cholesterol was actually the major lipid present, representing the only neutral lipid class in AcChR-rich membranes. In this regard, it is of interest that the material of choice for reconstitution of AcChR for several research groups (Epstein & Racker, 1978; Huganir et al., 1979; Nelson et al., 1980; Lindstrom et al., 1980) is commercial phospholipids from soybean (asolectin lipids) which contain significantly less sterols (phospholipid to sterol ratio ~9).[3] The use of asolectin lipids appears to provide a certain degree of activity in reconstituted systems which seems to indicate a less than critical role for cholesterol in receptor function. On the other hand, in a recent communication (Dalziel et al., 1980) the use of nonnative lipids supplemented with cholesterol in amounts approximating that found in electroplax membranes was reported to be necessary for optimal flux activity in AcChR-reconstituted vesicles. Additionally, Popot et al. (1978) described that in lipid monolayers the presence of cholesterol facilitated the interaction and subsequent incorporation of AcChR.

The most abundant phospholipid in AcChR-rich membranes was EP which was characterized by longer fatty chains and a greater degree of unsaturation than other phospholipids present. Presence of higher amounts of long polyunsaturated EP molecules in combination with a high cholesterol content might be of importance in providing special features to the membrane matrix associated with the in situ AcChR protein. Fluorescence results indicated a lower fluidity than in the media from AcChR-poor membranes, but also other membrane parameters such as the thickness of the bilayer could be effected by the abundance of the longer EP molecules.

The lipid vesicles provided a suitable system to separately study the lipid matrix associated with each of the native membrane fractions. Detergent dialysis from OG-solubilized lipid extracts produced large vesicles (about 2500 Å diameter) of comparable size to those obtained directly from electroplax tissue, eliminating surface curvature constraints present in smaller vesicle systems. This notion was supported by the similarity of the fluorescence dynamic data obtained from native membranes and from the same membranes solubilized with OG and then reconstituted.

The fluorophores DPH and TMA-DPH probe different membrane domains. Above the transition temperature, DPH is assumed to be located within the membrane core, aligned with the phospholipid acyl chains (Andrich & Vanderkooi, 1976). Conversely, the trimethylammonium head of TMA-DPH apparently interacts with the phospholipid polar head groups (Prendergast et al., 1981). To describe apparent fluidity, we have used a purely phenomenological parameter, the rotational relaxation time, which considers both the changes in fluorescence polarization and lifetime of the fluorophores (Gilmore et al., 1979). Also, as a complement to the above studies, we used the coumarin probe to study the differences induced at the membrane surface ionic environment by the presence of the different lipids and proteins in the native membrane system.

The results with DPH and TMA-DPH on vesicles assembled with the various lipid extracts suggested that the absence of discrete transitions in the lipid matrix within the experimental temperature range was due most likely to the highly unsaturated population of phospholipids even in the presence of high levels of cholesterol. Vesicles prepared with AcChR-rich membrane lipid extracts provided a more rigid environment than those from AcChR-poor membrane lipid. Additionally, the differences between their rotational relaxation time values can be explained almost solely on the basis of their different phospholipid to cholesterol molar ratios. Nevertheless, differences in phospholipid/cholesterol ratio do not explain the similar behavior between vesicles prepared with AcChR-rich and unfractionated membrane lipid.

A higher degree of order, probably due to the presence of protein, was observed in the native membranes than in vesicles assembled with complementary lipid extracts. In this regard, saturation transfer electron spin resonance techniques have not detected rotational motion for spin-labeled membrane-bound AcChR (Rousselet & Devaux, 1977). In addition, conventional electron spin resonance techniques produced a two-component spectra with a tightly immobilized component (Marsh & Barrantes, 1978; Andreasen & McNamee, 1980). Both types of electron spin resonance data suggest that a more rigid environment on the average should be observed in the AcChR-rich than in the AcChR-poor membranes. In fact, the degree of order appeared to be especially enhanced in the AcChR-rich membranes at the TMA-DPH domain which might be correlated to the high content of transmembranous AcChR which could affect phospholipid head-group arrangement [AcChR is the major integral protein in the electroplax membranes (Neubig et al., 1979; Klymkowski et al., 1980)]. The later argument received some support from the finding made on the titration of the coumarin probe which indicated a basic ionic environment provided by the lipids at the membrane surface. Lipid vesicles prepared from unfractionated, AcChR-rich, and AcChR-poor membrane lipid extracts exhibited apparent pK values of 9.35, 9.05, and 9.25, respectively. A slightly less basic pK for the coumarin probe in the vesicles prepared with AcChR-rich membrane lipid should be expected from the higher proportion of positively charged EP molecules. The presence of protein, however, in the native membranes appeared to negate the distinctive effects of the lipid on the apparent pKs. The increase in the coumarin pK for the AcChR-rich membranes (9.25) when compared to its corresponding lipid vesicles can be rationalized as due to the influence of the acidic pK of the receptor protein, lipid head-group rearrangements, or repartitioning of the probe itself into a different environment.

In conclusion, we have described the lipid environment of the AcChR as a membrane domain whose reasonably high degree of order appeared to be affected by both the presence of cholesterol and protein. Lipid compositional features associated with this AcChR-rich fraction were mainly the higher content of unesterified cholesterol and the presence of long-chain highly unsaturated ethanolamine phosphoglycerides composed of both PE and plasmalogens.

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