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ARTICLE *in* BIOCHEMISTRY · AUGUST 1982

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M. Criado, H. Eibl, and F. J. Barrantes

Biochemistry, **1982**, 21 (15), 3622-3629• DOI: 10.1021/bi00258a015 • Publication Date (Web): 01 May 2002

rative TLC and reconstituted mixtures containing one or more components from a different temperature regime or from microsomes are now feasible. Such studies should be useful in establishing the relative contributions of molecular species changes vs. changes in the overall fatty acid composition in defining the ciliary membrane's characteristic physical behavior.

Acknowledgments

We are grateful for the assistance of Jim Hudson with the GC–MS procedures.

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Effects of Lipids on Acetylcholine Receptor. Essential Need of Cholesterol for Maintenance of Agonist-Induced State Transitions in Lipid Vesicles†

M. Criado, H. Eibl, and F. J. Barrantes*

ABSTRACT: The effects of lipids on the maintenance of characteristic functional properties of the acetylcholine receptor during the course of reconstitution into lipid vesicles were studied by following the kinetics of agonist-induced state transitions. The requirements for successful preservation of these properties could be dissected into two components: (a) adequate nature and concentration of lipids during detergent solubilization; (b) correct lipid environment during reincorporation into lipid vesicles by the cholate dialysis procedure. Optimal bulk lipid concentrations and lipid:cholate ratios for preserving state transitions during solubilization were studied by using both crude soybean lipids and pure synthetic phospholipids. The latter class of lipids was found to be unsuitable substitutes for the crude soybean lipids, irrespective of their polar head group and/or fatty acyl chain, even when detergent:lipid ratios as high as 1:1 (w/w) were employed. Addition of cholesteryl hemisuccinate was able to make up this deficiency, attaining preservation of acetylcholine receptor state transitions at cholate:steroid ratios of about 6:1 (w/w). The presence of steroid decreased the amount of protein solubilized. The correct choice of lipid type was also essential to the reincorporation step, and higher concentrations of lipid were required—about 20 mg/mL for soybean lipids. Pure phospholipids at similar concentrations, however, were unable to maintain the state transitions. Again, steroid (40–46% cholesteryl hemisuccinate/mol of total lipid) provided the adequate conditions at the reincorporation stage and enhanced the amount of protein reincorporated into the vesicles. A large (70–90%) percentage of the receptor was reincorporated with the correct vectorial sidedness. No specificity could be detected for the phospholipid polar head or alkyl chain in relation to any of these findings. The effect of the protein on the physical state of the lipids in the reconstituted vesicles was studied by diphenylhexatriene fluorescence depolarization. The results may be interpreted as a disordering of the acyl chains in the gel state and an ordering in the liquid-crystalline state in the presence of protein, accompanied by shifts in the transition temperatures of the pure phospholipids to lower values.

The acetylcholine receptor (AChR)[1] is a multimeric protein complex of five subunits, two of which appear to be almost identical (α) and the other three (β, γ, δ) partially homologous (Raftery et al., 1980). Affinity labels react covalently with the α chain, suggesting that this polypeptide is related totally or partially to the cholinergic recognition site [see the review in Karlin (1980)]. The function of the other subunits remains unknown, although an involvement of the δ chain in the binding of noncompetitive blockers was suggested from affinity

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[1] Abbreviations: AChR, acetylcholine receptor; DMPC, L-β,γ-dimyristoyl-α-lecithin; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphate; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; PMPC, 1-palmitoyl-2-myristoyl-*sn*-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; NP buffer, 100 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride, and 10 mM sodium phosphate buffer, pH 7.4; DPH, 1,6-diphenyl-1,3,5-hexatriene.

0006-2960/82/0421-3622$01.25/0

label experiments using a local anesthetic derivative (Saitoh et al., 1980; Oswald & Changeux, 1981). This polypeptide is also responsible for the covalent association of AChR monomers into dimers (Chang & Bock, 1977; Hamilton et al., 1979).

The AChR is an intrinsic membrane protein. This is substantiated by the application of physicochemical criteria for the classification of membrane proteins (Barrantes, 1975; Raftery et al., 1980; Cantor & Schimmel, 1980) and the results of labeling experiments in which the α subunit (Tarrab-Hazdai et al., 1980) or the β and γ subunits (Sator et al., 1979) can be reached from the membrane phase. Similarly, biochemical (Huang, 1979; Wennogle & Changeux, 1980; Strader & Raftery, 1980) and immunological (Tarrab-Hazdai et al., 1978) data suggest that the AChR transverses the membrane. Thus, the lipid environment may be an important modulator of AChR properties. In fact, the delipidation of the AChR modifies its affinity for agonists (Briley & Changeux, 1978; Chang & Bock, 1979), and the treatment of the membranes with phospholipase A_2 affects the ion translocation (Andreasen et al., 1979). Further, measurement of integrated flux responses to agonist binding in reconstituted systems has become an established analytical tool in receptor research since Epstein & Racker (1978) pointed out the need to maintain an elevated lipid content in the course of AChR reconstitution. Since then, several other laboratories have successfully adopted the essential aspects of Racker's strategy (Wu & Raftery, 1979; Lindstrom et al., 1980a; Anholt et al., 1981; Changeux et al., 1979; Sobel et al., 1980). This has opened the way for the study of the interrelationships between the AChR and its lipid environment under defined conditions.

Exposure of the AChR to certain cholinergic ligands, notably nicotinic cholinergic agonists, results in concentration- and time-dependent changes in the affinity state of the receptor for such ligands (Weber et al., 1975; Weiland et al., 1976; Barrantes, 1976; Colquhoun & Rang, 1976). The apparent rate of AChR–toxin complex formation provides an indirect but sensitive measure of these changes in the second-to-minute time domain. The affinity transitions are characteristically observed in the membrane-bound AChR preparations and are normally lost upon solubilization in anionic or nonionic detergents, unless the latter are supplemented with exogenous lipids (Heidmann et al., 1980a). The ability to undergo ligand-induced state transitions appears therefore to be an environmentally sensitive property of the AChR, and the toxin rate measurement appears to be a useful criterion of its maintenance.

The present work is concerned with the requirements for preserving the affinity transitions of the AChR during (a) its solubilization in sodium cholate and (b) its subsequent incorporation in lipid vesicles of pure synthetic lipids with or without cholesterol. The presence of lipids during the detergent solubilization process is found to be essential. A dicotomy between the protective effects of lipids during steps a and b above is made on the basis of their relative contributions to the maintenance of AChR state transitions. The presence of exogenous cholesterol appears to play a decisive role in the preservation of the agonist-induced state transitions of the AChR, an effect that is exerted during both detergent solubilization and reincorporation into lipid assemblies.

Experimental Procedures

Materials

L-β,γ-Dimyristoyl-α-lecithin (DMPC) (99%) was from Fluka AG, Buchs GS, Switzerland; cholesteryl hemisuccinate (CHS) (+99%) was from Platz & Bauer, Stanford, CA; crude soybean lipids ("L-α-phosphatidylcholine") were from Sigma, Munich, West Germany; sodium cholate was obtained from Serva, Heidelberg, West Germany. The synthesis of 1,2-dimyristoyl-*sn*-glycero-3-phosphate (DMPA) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) was reported by Eibl & Woolley (1979) and Harlos & Eibl (1980), respectively. The mixed-acid phospholipids 1-palmitoyl-2-myristoyl-*sn*-glycero-3-phosphocholine (PMPC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) were prepared according to Eibl (1980) via the corresponding bromoethyl esters. The positional purity of the fatty acid esters in the 1 and 2 position of the glycerol molecule exceeded 99% in all cases. Phosphate was exclusively attached to the 3 position as indicated by the ratio of phosphate (Eibl & Lands, 1969) to vicinal diol (Eibl & Lands, 1970) of 1:1 as described for the positional analysis of phospholipids (Eibl & Lands, 1982). Native α-bungarotoxin was from Miami Serpentarium, Miami, FL, and its tritium derivative (sp act. 48 Ci/mmol) from Amersham Buchler, Braunschweig. α-Cobrotoxin from the venom of *Naja naja atra* (K & K Laboratories) was labeled as in Barrantes (1978). *Torpedo marmorata* electric fish were obtained from the Marine Biological Station at Arcachon, France. AChR-rich membrane fragments were prepared as described by Barrantes (1982).

Methods

Solubilization of AChR-Rich Membranes. AChR-rich membranes were dissolved in 2% sodium cholate (10 mg of detergent/mg of protein) in the presence or absence of the indicated concentrations of lipids in 100 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride, and 10 mM sodium phosphate buffer, pH 7.4 (NP buffer). The membranes were shaken for 90–120 min at 4 °C. Nonsoluble material was removed by centrifugation in an airfuge for 5 min at maximal speed.

Incorporation of AChR into Lipid Vesicles. Lipid concentrations were adjusted before and/or after the solubilization of AChR-rich membranes by several procedures: (a) Soybean lipids were dissolved in chloroform, and the solvent was evaporated under N_2 and dried in vacuo for 2–3 h at 4 °C. The residue was dissolved in 2% sodium cholate (20 mg of lipid/mL) and submitted to two cycles of freezing and thawing, after which the solution was completely clear. (b) DMPC, PMPC, SOPC, and DMPG were dissolved as the soybean lipids, but the freezing–thawing cycles were not necessary. (c) DMPA and POPE were suspended in 1% sodium cholate (20 mg of lipid/mL) and sonified to clarity (5–10 min) with a Branson sonifier at 40 W. (d) CHS was dissolved in 5% sodium cholate at pH 11.5 (26 mg/mL). A lower pH was ineffective in solubilizing CHS in cholate. The pH was thereafter readjusted to 7.4, the lipid remaining in solution. The different reconstitution mixtures, containing in each case a final concentration of 2% sodium cholate, 2% exogenous lipids, and the solubilized proteins in NP buffer, were dialyzed 2 times for 48 h against 500 volumes of NP buffer. The dialyzed material was diluted with buffer and centrifuged in an airfuge at maximal speed. The vesicles were resuspended in NP buffer (0.5–1 mg of protein/mL) and assayed as described below.

Sucrose Density Gradient Centrifugation of Reconstituted Vesicles. Reconstituted vesicles containing, on average, 80 μg of protein and 1500 μg of lipid (see Table IV) in a total volume of ~120 μL of NP buffer were supplemented with trace amounts of [^{3}H]-α-bungarotoxin and incubated overnight at 4 °C. They were then applied atop 5.2 mL of 5–30% (w/v)

Table I: Association of AChR with Soybean Lipids[a]

solubilization step			incorporation step			% initial rate[e]	
soybean lipid concn (mg/mL)	exogenous: endogenous lipid ratio	protein solubilized (%)[b]	protein incorporated (%)[c]	% AChRs oriented right side out[d]	lipid:protein ratio (w/w)	carb added simultaneously	carb preincubated
0		66.0	73.5	75.2	8.9	71.6 ± 7.1	59.1 ± 5.7
1.63	1.06	55.0	72.0	85.7	10.6	48.4 ± 6.8	13.5 ± 0.5
20.0	13.0	49.5	76.2	76.3	12.1	34.6 ± 3.6	17.8 ± 3.3

[a] AChR membranes solubilized in 2% sodium cholate and the indicated amount of soybean lipids. [b] Expressed as percentage of the initial amount of protein present in the membranes. [c] Expressed as percentage of the solubilized protein recovered in the reconstituted vesicles. [d] Determined according to Hartig & Raftery (1979). [e] Calculated from the apparent association rate constant as indicated under Methods. Expressed as percentage of the corresponding controls in the absence of agonist ± standard deviation. Values were 74.6% and 1.7%, respectively, in native membranes. Duplicate experiments were performed in all cases.

Table II: Effect of Lipids on Preservation of Ligand-Induced Transitions during Solubilization and Incorporation of the AChR

lipid present during			% initial rate[d]	
cholate:lipid ratio (w/w)	solubilization[a]	incorporation[b]	Carb added simultaneously	Carb preincubated
	none	DMPC (20 mg/mL)	84.0 ± 5.3	65.6 ± 11.9
1	DMPC (20 mg/mL)	DMPC (20 mg/mL)	65.8 ± 6.3	56.6 ± 4.1
12	soybean lipids (1.625 mg/mL)	92% DMPC, 8% soybean lipids[c]	72.5 ± 3.3	41.6 ± 7.5
12	soybean lipids (1.625 mg/mL)	92% PMPC, 8% soybean lipids	69.9 ± 1.8	40.4 ± 10.0
	none	65% DMPC, 35% CHS	78.3	62.8
6	CHS (3.25 mg/mL)	65% DMPC, 35% CHS	63.1	7.6
1	35% CHS, 65% DMPC (20 mg/mL)	65% DMPC, 35% CHS	31.8 ± 4.6	19.2 ± 6.7

[a] AChR-rich membranes (800 μg of protein, 2–3 nmol of α-toxin sites) were dissolved as indicated under Methods. Concentrations of lipids in the cholate extract are given. [b] After removal of the nonsolubilized material by centrifugation, the lipid composition and concentration were adjusted, if necessary, to 20 mg/mL, and the formation of liposomes was performed by dialysis as under Methods. [c] The composition of the final lipid mixtures is indicated in percentages (weight/weight). [d] Same criteria as in Table I and the text.

linear sucrose gradients and centrifuged in a Beckman SW 50.1 rotor at 50 000 rpm for 5 h. Radioactivity, lipid phosphorus, and refractive index measurements were carried out on 150-μL fractions.

Agonist-Induced Affinity Transitions. The ligand-induced state transitions of the reconstituted AChR were measured in the presence or absence of 1 μM Carb added simultaneously or 30 min before the addition of [^{3}H]-α-cobrotoxin as in Barrantes (1978). Calculation of the kinetic parameters was carried out in a UNIVAC 1108 computer as described (Barrantes, 1978).

Fluorescence Measurements. The fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) as a function of temperature was measured with a spectrofluorometer constructed in this laboratory. A Glan-air polarizer prism (Steig & Reuter, Frankfurt, West Germany) was used in the excitation beam, and a Kaesseman thin-film polarizer was used in the emission path. Zone-purified DPH (a gift from Dr. M. Shinitzky, The Weizmann Institute, Rehovot, Israel) was dissolved in tetrahydrofuran at a final concentration of 2 mM. Aliquots of the stock solution were diluted with NP buffer to a final concentration of 2 μM and added to the reconstituted vesicles to give a ratio of 1 DPH molecule for about 300 lipid molecules. After an incubation of 1 h at 30 °C the fluorescence was measured (360-nm excitation, 430-nm emission). Anisotropy parameters were calculated as in Shinitzky & Barenholtz (1978).

Other Analytical Procedures. Protein concentration and toxin binding activity of the membranes were determined as described by Lowry et al. (1951) and Schmidt & Raftery (1973), respectively. The sidedness of the AChR vesicles was determined as described by Hartig & Raftery (1979). Cholesterol content was assayed as reported by Huang et al. (1961), and phosphorus determinations were carried out according to Ames & Dubin (1960).

Results

Two main processes can be distinguished in the reassembly of the AChR in lipid vesicles: (i) the separation of the AChR from the native membranes by solubilization with detergents and (ii) the incorporation of the protein into the host bilayer. In practice, this can be accomplished in the formation of AChR-containing lipid vesicles by dialysis of the detergent-solubilized material [the detergent dialysis technique of Kagawa & Racker (1971)]. In the present work these two processes have been separately explored in order to determine the conditions that preserve the agonist-mediated affinity transitions of the AChR.

Effects of Lipids in Solubilization Step. In the native membranes used in this study, the presence of 1 μM Carb added simultaneously with the toxin or preincubated for 30 min reduced the apparent association rate constant to 74.6% and 1.7%, respectively, of the original value without agonist (Table I). As can be seen in Figure 1 and Table I, solubilization of the membranes in 2% sodium cholate irreversibly stabilized the AChR in a low-affinity state. The presence of soybean lipids in a detergent:lipid ratio of 12:1 (w/w) during the solubilization step preserved the agonist-induced transitions of the AChR. Higher soybean lipid concentrations, like those used in the subsequent incorporation step, were equally effective although the degree of AChR solubilization slightly diminished (Table I). It is noticeable that even when the presence of lipid preserved the AChR state transitions, a small proportion of the AChR still remained in the low-affinity state in the presence of ligand; this was not the case for the native membranes, where all the AChR was interconvertible with a 30-min preincubation.

The mere presence of phospholipids in the solubilization step

Table III: Effect of Increasing Concentrations of Cholesteryl Hemisuccinate on Agonist-Induced Transitions of the AChR Incorporated into DMPC–CHS–Liposomes

CHS during solubilization (mg/mL)[a]	CHS in reconstituted vesicles (mol %)[b]	lipid:protein ratio (w/w)	% incorporated protein[c]	% initial rate[d]	
				Carb added simultaneously	Carb preincubated
1.1	13.9	11.74	81	78.4 ± 2.8	76.6 ± 5.5
3.3	31.2	12.52	82	74.5 ± 5.7	71.9 ± 3.1
5.6	46.2	16.56	86	31.8 ± 4.6	19.2 ± 6.7
7.8	57.0	22.00	74	88.1	39.5

[a] AChR-rich membranes were solubilized in 2% sodium cholate containing 20 mg of DMPC plus CHS/mL at the concentrations given in the left column. The soluble extract was dialyzed as under Methods. [b] The concentration of CHS was determined after dialysis and differed only slightly from the values obtained prior to dialysis. [c] Normalized values; 100% represents the total protein amount offered for reconstitution. [d] The affinity transitions of the AChR were tested as in Tables I and II. Values are expressed as percentage of the corresponding controls in the absence of agonist ± standard deviation.

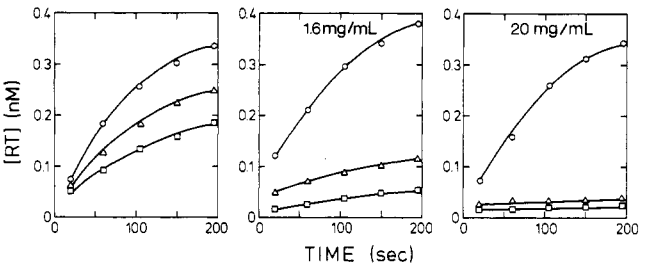

FIGURE 1: Kinetics of AChR–[^{3}H]-α-cobrotoxin association of AChR incorporated into soybean lipid vesicles. AChR membranes (800 μg of protein, 2.4 nmol of α-toxin sites) were solubilized in 2% sodium cholate containing no exogenous lipid (left panel) or soybean lipids at the concentrations indicated in the figure. After 90 min the soluble extract was supplemented if necessary with additional soybean lipids for the reconstitution (see Experimental Procedures). Toxin rate assays were carried out at 22 °C in the absence (○) or presence of 1 μM Carb given either simultaneously (△) or 30 min before (□) toxin addition: $[R_0]$ = 2.25 nM; $[T_0]$ = 9 nM.

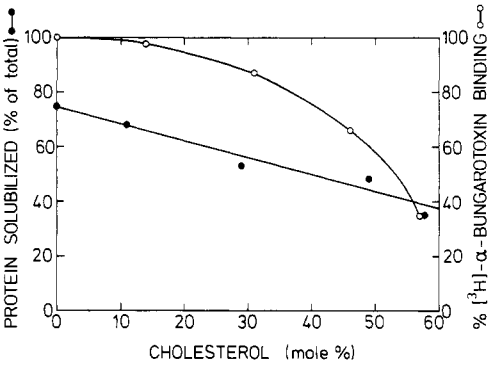

FIGURE 2: Effects of CHS on solubilization of AChR membrane proteins and α-toxin equilibrium binding of incorporated AChR. AChR membranes (concentration as given in Figure 1) were solubilized in 2% sodium cholate containing 20 mg/mL of a 2% DMPC + CHS mixture. The initial molar ratios of CHS in the mixture were 0%, 11%, 29%, 49%, and 58%. The soluble extract was dialyzed, and the resulting vesicles were separated by centrifugation as given under Experimental Procedures.

did not suffice for the conservation of the agonist-induced transitions, even at lipid:detergent ratios as high as 1:1 (w/w). This is illustrated for DMPC in Table II. Further, the inability to preserve the affinity transitions was not exclusive of this phospholipid (Table IV).

Lipid Requirement in Incorporation Step. The question then arose as to whether the effects of soybean lipids during the solubilization process are also exerted while the AChR is being incorporated into the lipid vesicles. This was tested by using the same concentrations of soybean lipids able to protect the AChR affinity transitions in the first process (solubilization), except that the lipids used in the second stage (incorporation) were PMPC–soybean and DMPC–soybean lipid mixtures (Table II). In neither case could the AChR reach the high-affinity state with Carb preincubation (Table II), suggesting that the soybean lipids were necessary not only to protect the AChR in detergent solution but also during its assembly into lipid vesicles. When lipid concentrations like those used by Anholt et al. (1981) in their flux assay were tested, preservation of affinity state transitions was observed (Table I).

Effects of Cholesteryl Hemisuccinate. Synthetic phospholipids by themselves could not replace the crude soybean lipids in their protective effects (Table II). Since Kilian et al. (1980) had already noticed the requirement of neutral lipids in the soybean crude mixture and cholesterol is a major constituent of the AChR membranes (Popot et al., 1978), we studied the effects of CHS on the above properties. From the results presented in Table II it is evident that only when CHS was present throughout the duration of the two processes were the affinity transitions preserved. As occurred with the soybean crude lipids, the presence of cholesterol was essential during solubilization, a cholate:CHS ratio of 6:1 (w/w) being sufficient to protect the solubilized receptor. The same result was obtained when the phospholipid–cholesterol mixtures used in the subsequent incorporation (see Table II) were already present in the solubilization step. However, the concentration of CHS necessary to protect the AChR during solubilization, and even slightly higher, was not sufficient to exert any protection in the incorporation step (Table III).

Increasing concentrations of CHS in contact with the native membranes lowered the percentage of protein solubilized (Figure 2). This was accompanied by a slight inhibition of the [^{3}H]-α-bungarotoxin equilibrium binding (Figure 2), more pronounced at the maximal CHS concentration tested. The protein incorporation was not markedly affected (Table III). Optimal incorporation took place at a concentration of 46 mol % of CHS, which is approximately the proportion of cholesterol in the native membranes (Popot et al., 1978). Of the four concentrations tested, 46 mol % was also the CHS concentration necessary to observe the affinity transitions of the AChR (Table III).

Lack of Influence of Phospholipid Head Group or Acyl Chain Composition. The effects of various phospholipids differing in their polar heads and/or fatty acyl chains were tested next in both the solubilization and incorporation steps, in the presence and absence of CHS (Table IV and Figure 3). The solubilization of protein was not markedly affected by the nature of the lipid present in this process. The two extreme cases were POPE and DMPG; the protein solubilized in the presence of the former was 75% of that recovered in the

Table IV: Association of AChR with Cholesteryl Hemisuccinate and Synthetic Phospholipids of Different Polar Heads and/or Fatty Acid Composition

lipid[a]	protein solubilization (%)[b]	protein incorporation (%)[c]	% AChR oriented right side out	lipid:protein ratio (w/w)	CHS (mol %)	% initial rate[e]	
						Carb added simultaneously	Carb preincubated
SOPC	62.2	60.0	90.0	5.7		86.3	81.3
SOPC, CHS	47.4	65.3	87.2	23.3	49.7	74.2 ± 6.8	55.9 ± 4.9
PMPC	65.0	75.8	77.6	16.0		73.4 ± 2.5	60.4 ± 8.2
PMPC, CHS	39.0	81.7	74.8	16.5	42.6	71.7 ± 7.6	23.0 ± 5.1
POPE	54.0	84.8	71.4	19.7		81.6	49.8
POPE, CHS	43.8	90.4	72.8	21.6	39.2	55.6 ± 1.3	13.6 ± 1.5
DMPG	71.1	84.6	91.5	8.3		93.5	87.6
DMPG, CHS	35.0	96.0	84.6	11.8	46.9	75.0 ± 3.2	28.0 ± 3.2
POPE, SOPC, DMPG, CHS[d]	25.0	76.5	80.0	30.1	45.6	25.3	9.7
POPE, SOPC, DMPG, CHS[d]	32.0	52.0	70.4	28.3	46.4	40.2	15.6

[a] When CHS was present, its initial concentration was in each case 35% (w/w) of the total exogenous lipids. [b] Solubilization is expressed as percentage of the initial protein present in the membranes. All the lipid used for the incorporation was already present in the solubilization process. [c] Incorporation is expressed as percentage of the solubilized protein recovered in the reconstituted vesicles. [d] The initial composition (by weight) of these mixtures was 26% POPE, 26% SOPC, 13% DMPG or DMPA, and 35% CHS. [e] Agonist-induced affinity transitions of the incorporated AChR were assayed as in Tables I–III.

presence of the latter (Table IV). As already observed for DMPC (Figure 2), the presence of CHS lowered in each case the percentage of protein solubilized, but in contrast, the percentage of protein incorporated was enhanced.

A direct demonstration of the formation of AChR–lipid complexes was obtained from sucrose gradient centrifugation experiments. As shown in Figure 3, the distribution of toxin binding activity and lipids was found to coincide within a wide range of lipid:protein ratios and to be independent of the lipid nature. Only the relative position of the peaks along the gradient varied with the relative amounts of lipid and protein and with the presence or absence of cholesterol (Figure 3). No correlation was found between the chemical nature of the phospholipid used and the percentage of AChR oriented right side out, a wide variability (70–90%) being observed. The final percentage of CHS incorporated in the vesicles varied slightly for different phospholipids.

The physical state of the lipid did not appear to influence the AChR affinity transitions. Four different phosphatidylcholines with transition temperatures of 5 °C (SOPC), 17 °C (1-stearoyl-2-lauryl-*sn*-glycerophosphocholine, SLPC, data not shown), 24 °C (DMPC), and 28 °C (PMPC) yielded similar results (Table IV). Variations in the polar head of the phospholipid had some influence on the affinity transitions (Table IV). POPE elicited the larger span in toxin rates with and without agonist incubation, although the absolute values still represented a small percentage of what is observed in the native membranes. A marked improvement in the depression of toxin rates was attained upon addition of CHS to the phospholipids and in particular to mixtures thereof resembling the proportion of naturally occurring phospholipids (Table IV).

Effects of AChR Protein and CHS on Phospholipid Thermal Behavior. The association of the polypeptides of the AChR-rich membranes with the different lipids tested was also studied by fluorescence depolarization of DPH, a specific probe for the hydrophobic region of the lipid bilayer and sensitive to the gel to liquid-crystalline transitions of the lipids. The transition temperatures of DMPC, DMPG, PMPC, and POPE, as detected by steady-state DPH fluorescence anisotropy, occurred at 24.5, 32.5, 28, and 27.5 °C, respectively. A shift of about 1 °C below the above temperatures was observed in each case in vesicles containing protein in addition to the pure lipid (Figure 4). When CHS was present in concentrations between 40 and 47 mol %, the sharp transition was no longer observed. No additional effect of the protein

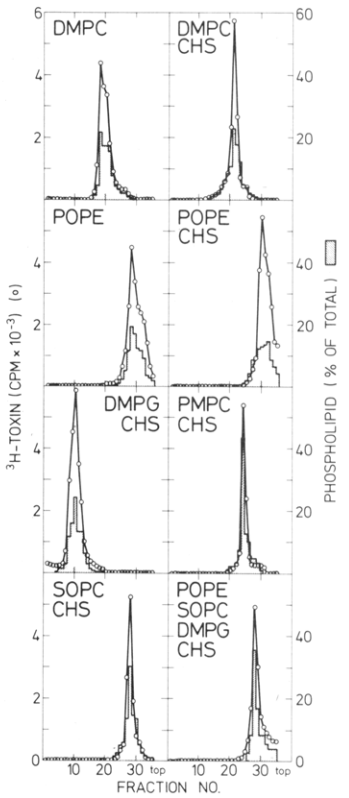

FIGURE 3: Sucrose gradient centrifugation of AChR reconstituted into lipid vesicles (composition indicated in each case). Lipid:protein ratios were as given in Table IV. Toxin binding activity and lipid phosphorus were analyzed in 150-μL aliquots as described under Experimental Procedures. Total phospholipid refers to the amount applied to each gradient.

could be detected in phospholipid–CHS–protein vesicles (Figure 4). In the case of AChR–synthetic phospholipid vesicles there was a decrease in the fluorescence anisotropy of DPH in the DMPC and PMPC gel state (parts A and C of Figure 4). This was also true for SOPC and SLPC above the phase transition temperature (data not shown) and suggests that the protein disrupts the ordering of the fatty acyl chains in the gel state. In the case of DMPG and POPE this effect was not clearly observed (parts B and D of Figure 4). Above the transition temperature there was an increase in the anisotropy of DPH, suggesting that the presence of protein lowered the enthalpy change and ordered the fatty acyl chains

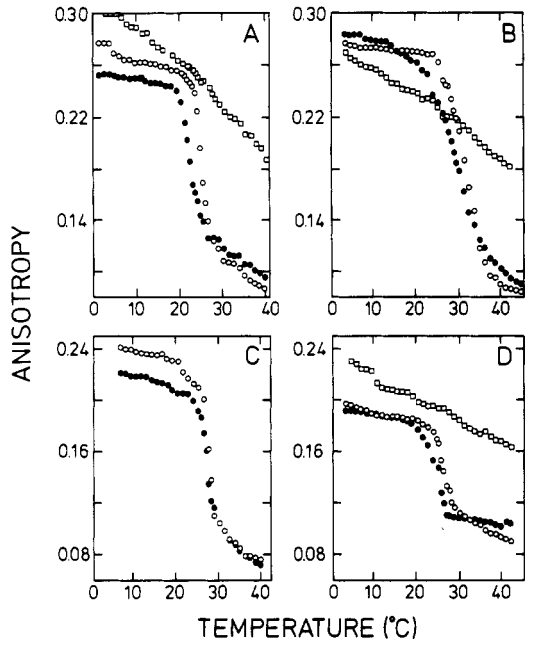

FIGURE 4: Fluorescence anisotropy of DPH in lipid vesicles with or without AChR or cholesterol. The probe was incorporated in pure DMPC (A), DMPG (B), PMPC (C), or POPE (D) [(○) in all cases] or in the same host lipid vesicles supplemented with protein and CHS (□) at final concentrations of the latter of 45.8 (A), 46.9 (B), and 39.2 mol % (C). The temperature-dependent anisotropy in the phospholipid vesicles containing AChR protein (●) is also shown in each case.

in the liquid-crystalline state. This was also apparent with soybean lipids (not shown).

Discussion

It is now recognized that the experimental conditions commonly used in the past for solubilization and purification of the AChR result in the loss of the characteristic kinetic properties present in the membrane-bound state [for reviews, see Briley & Changeux (1978) and Barrantes (1979)]. One possible reason for the stabilization of the AChR in a low-affinity state (different from the one capable of undergoing state transitions) upon solubilization in detergents emerged from a series of observations from Racker's laboratory (Epstein & Racker, 1978; Huganir et al., 1979; Kilian et al., 1980) on reconstitution of agonist-mediated fluxes. These observations made apparent the need to carry out the reconstitution of the AChR in detergent supplemented with exogenous lipid in order to preserve the integrated $^{22}Na^+$ translocation. Similarly, reports from Changeux's laboratory have followed this criterion for the successful preservation of the agonist-responsive low-affinity state of the AChR, as measured indirectly with a fluorescent agonist (Changeux et al., 1979; Heidmann et al., 1980a,b; Sobel et al., 1980).

The first attempts to dissect the relative contributions of the constituents in the crude soybean lipids ("asolectin") can be found in Kilian et al. (1980). Various phosphoglycerides were found inadequate to substitute the mixed soybean lipids in the reconstitution of flux activity (Kilian et al., 1980), as shown here for the preservation of agonist-induced state transitions. In the present case a more extensive screening of the phospholipid nature has been attempted with pure, synthetic phospholipids. Variations of the acyl chain length (C_{12} to C_{18}) in choline phosphoglycerides or changes in the polar head region (ethanolamine, choline, glycerol) affecting size, charge, and other physicochemical properties of the lipids (see below) were tested. The basic outcome of this part of the study is that phosphoglycerides alone, without additional neutral lipids, are inadequate substitutes for the mixed soybean lipids, independently of their polar heads and/or acyl chain compositions (Tables II and IV).

Kilian et al. (1980) also noticed that excitability in AChR–lipid vesicles is strongly dependent on the presence of neutral compounds. No attempts were made, as in the present case, to correlate the preservation of function with the presence of high contents of (endogenous) cholesterol, which is a distinct characteristic of the AChR membranes (Popot et al., 1978). Here, the contribution of cholesterol to the maintenance of AChR state transitions is made apparent (Tables II–IV). It is further shown to be independent of the chemical and physical properties of the accompanying phospholipid (Tables II and IV). Dalziel et al. (1980) demonstrated that cholesterol was necessary for the maintenance of the ion flux properties of reconstituted AChR. In their experiments, cholesterol appeared not to affect the ligand binding properties of the AChR nor its ability to undergo affinity transitions. The suggestion was made that cholesterol was without effect on the latter properties, in contrast to the present findings.

The second novel aspect of the present work concerns the distinction between two separate processes in which the lipid protective effect is operative. It is already clear from the experiments with crude soybean lipids that they are necessary not only to "protect" the AChR during its solubilization in detergent but also to provide an adequate environment to the AChR during its incorporation into the host lipid vesicle (Table II). Again, pure phospholipids like DMPC and PMPC are unable to fulfill this requirement.

About 60% of the protein in AChR membranes can be extracted with 2% cholate in the presence of soybean lipids. The concentration of soybean lipids in the solubilization step [1:10 lipid:cholate (w/w)] necessary to preserve the AChR channel activity (Anholt et al., 1981) is sufficient for the protection of the AChR in terms of its affinity transitions (Table I). Huganir et al. (1979) reported that solubilization with low cholate concentrations (<1%) allowed endogenous lipids to preserve the integrated $^{22}Na^+$ flux activity without the need for exogenous lipids. However, under such conditions suboptimal extraction of the AChR occurs.

In agreement with Anholt et al. (1981), more lipid is required to successfully accomplish the reincorporation of the AChR into the lipid vesicles. These results suggest different physicochemical requisites at each step. Solubilization can be envisaged as a disruptive step, in which an essentially two-dimensional lattice (the membrane) is fragmented into smaller, mixed micelles of protein, lipid, and detergent. Essentially the same physical processes govern the formation of micelles of ionic or nonionic surfactants (Wennerstroem & Lindmann, 1979). The micelles of bile salts and choline phosphoglycerides are a case apart, since neither cholate nor lecithins having more than 10 carbons in their alkyl chains form proper micelles individually (Lindman & Wennerstroem, 1980) but aggregate directly to a lamellar liquid-crystalline structure (Wennerstroem & Lindmann, 1979). The mixed systems are nevertheless typical micellar aggregates. Dervichian (1968) proposed that these consist of disks of lecithin, whose hydrophobic edges are covered by cholate molecules. The size of the mixed micelles augments as the phospholipid:detergent ratio increases (Helenius & Simons, 1975), a factor to be taken into account when the "protective" effects of lipids on the AChR are considered. In the reverse process (the reincorporation step) the additional phospholipid offered to the mixed micellar system should progressively stabilize the lamellar structure of the vesicles, which can exist in the

presence of excess water (Israelachvili et al., 1976). We can only surmise that the AChR reincorporation into the vesicular structure is a favored process, as evidenced by the amounts of protein recovered in the resulting liposomes (Tables II and III).

The ability of a pure steroid to substitute the total neutral components in soybean lipids is evidenced by our experiments. Sodium cholate, when supplemented with CHS [cholate:CHS, 6:1 (w/w)], was effective in solubilizing receptors while maintaining their agonist-induced affinity transitions (Table II). The detailed mechanism of the protection effect exerted by CHS in detergent solution remains to be elucidated, but in a first approximation one could envisage a mutual competition of cholesterol and cholate in their interaction with the immediate environment of the AChR. The common cyclopentylphenanthrene ring provides a basis for structural similarities between sodium cholate and cholesterol. A high exogenous cholesterol content might prevent the deleterious action of the structurally related detergent by inhibiting the displacement of endogenous cholesterol. The solubilizing action of cholate should therefore be diminished in the presence of high amounts of exogenous CHS, and this appears to be the case. This hypothesis is supported by the observation that bile salts solubilize cholesterol rather poorly (Carey & Small, 1970). Also, solubilization of cholesterol from erythrocyte membranes by deoxycholate begins to occur only after 60% of the protein and phospholipid have been solubilized (Kirkpatrick et al., 1974).

Amounts of CHS effecting optimal protection of the AChR in the course of its solubilization (Table II) did not suffice to accomplish an equivalent degree of preservation of the affinity transitions through the incorporation step (Table III). The condensing effect of cholesterol on the packing of phospholipid chains is well-known [for a review, see Demel & De Kruiff (1976)]. Native AChR membranes are extremely rigid, probably as a consequence of their high cholesterol content (Popot et al., 1978) and high protein:lipid ratio. In the reconstituted system, cholesterol could exert similar effects toward the mimicking of the natural environment. The incorporation of protein was enhanced by the presence of CHS (Tables III and IV). Increased incorporation of AChR protein into monolayers containing cholesterol, in comparison to those made of phospholipids alone, has been reported (Popot et al., 1978). These effects do not necessarily imply, however, any selective affinity of the AChR for cholesterol.

The apparent initial rate of α-toxin binding in reconstituted vesicles was always lower than that in control, native membranes, especially under conditions of simultaneous toxin plus agonist addition (Tables I–IV). These data indicate that the presence of exogenous lipids does not only protect the affinity transitions but also may accelerate them. The inhibition of the equilibrium binding of α toxin upon increasing the CHS concentration (Figure 2) is puzzling, because it already occurs at CHS:phospholipid ratios below those found in the native membrane (Popot et al., 1978). One should bear in mind, however, that the protein:lipid ratios in the reconstituted vesicles are much lower than those found in the latter case. Protein–protein interactions, dominating in the native membrane, are thus minimized in the reconstituted systems. Under such conditions tocopherol concentrations above 40% have been found to be "toxic" for the gating function of the AChR (Kilian et al., 1980). Such detrimental action is in agreement with the present observations.

The high proportion of right-side-out assembled receptor, independent of the lipid forming the vesicles, suggests a certain capacity of the protein to organize its environment and/or to facilitate its self-assembly. This may in turn result from the inherent asymmetry of the AChR molecule, in terms either of its subunit composition (Lindstrom et al., 1980b) or of its morphology (Zingsheim et al., 1980). This natural ability of the AChR to correctly assemble in the lipid bilayer appeared to be independent of the phospholipids present (Tables III and IV). This lack of specificity is in agreement with the electron spin resonance experiments of Marsh & Barrantes (1978) and Marsh et al. (1981), in which the selective immobilization of a substantial proportion of the lipids in AChR native membranes was reported. Though selective, the immobilized lipid annulus in the immediate vicinity of the AChR affected equally steroid, phospholipid, and fatty acids.

Although the interpretation of the steady-state fluorescence anisotropy of DPH in terms of the so-called microviscosity is still a matter of controversy (Kagato et al., 1978; Hildenbrand & Nicolau 1979), such measurements provide information on the structural order in membranes. The effects of the AChR protein on the DPH depolarization suggest that the lipid layers interact with the protein in such a way that a disordering of the acyl chains occurs in the alkyl chain gel state and ordering in the liquid-crystalline state. The shifts in the transition temperatures observed in the presence of AChR protein (Figure 4) are analogous to those observed in other systems (Petri et al., 1981; Chapman et al., 1974). Cholesterol, as expected, suppressed the sharp transition enthalpy and produced a spread, continuous temperature-dependent change (Figure 4). In the case of the ternary systems phospholipid–cholesterol–protein the effects of the latter constituent did not make themselves apparent in the DPH fluorescence anisotropy (Figure 4). It has already been noticed that at cholesterol: phospholipid ratios above 0.5 the effects of proteins become less noticeable (Shinitzky & Inbar, 1976).

The preservation of the lipid environment appears to play a decisive role in the maintenance of $^{22}Na^{+}$ flux and agonist-induced affinity transitions of the AChR reconstituted in vesicular systems. This lipid requirement may differ from the requisites for successfully reconstituting the AChR in planar lipid bilayers, where a pure synthetic phospholipid was equally effective as host lipid with or without additional endogenous cholesterol or phospholipids (Boheim et al., 1981). Furthermore, caution should be exercised in comparing the criteria used in planar lipid bilayer experiments with those of measurements of integrated, bulk properties like flux or affinity transitions. Thus the enhanced incorporation of AChR protein in CHS-containing vesicles reported here may bear a relation to the analogous preference of AChR for cholesterol-rich monolayers (Popot et al., 1978), and the affinity transition properties may, in turn, be related to the reported changes in gating behavior of the AChR in planar bilayers as a function of cholesterol content (Schindler & Quast, 1980). It is unlikely, however, that the influence of cholesterol on surface pressure in planar bilayers (Schindler & Quast, 1980) can be rationalized on the same molecular basis as the AChR agonist-induced state transitions.

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