A Minimum Number of Lipids Are Required To Support the Functional Properties of the Nicotinic Acetylcholine Receptor†

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ABSTRACT: The detergent sodium cholate was used to both solubilize and partially delipidate the nicotinic acetylcholine receptor from *Torpedo californica*. Using both native membranes and reconstituted membranes, it is shown that the detergent to lipid molar ratio is the most important parameter in determining the effect of the detergent on the functional properties of the receptor. Receptor–lipid complexes were quantitatively separated from detergent and excess lipids by centrifugation through detergent-free sucrose gradients. The lipid to protein molar ratio of the complexes could be precisely controlled by adjusting the cholate and lipid concentrations of the starting membranes. Analyses of both ion influx activity and ligand binding revealed that a minimum of 45 lipids per receptor was required for stabilization of the receptor in a fully functional state. Progressive irreversible inactivation occurred as the lipid to protein mole ratio was decreased below 45, and complete inactivation occurred below a ratio of 20. The results are consistent with a functional requirement for a single shell of lipids around the perimeter of the receptor.

The nicotinic acetylcholine receptor (AChR)[1] is an integral membrane protein which mediates synaptic transmission at the vertebrate neuromuscular junction and the electric organ synapses of certain electric fish [for reviews, see Barrantes (1983), Popot and Changeux (1984), and McCarthy et al. (1986)]. In recent years, the receptor has been purified and successfully reintegrated into both lipid vesicles (McNamee & Ochoa, 1982; Jones et al., 1987) and planar bilayers (Labarca et al., 1985) where it exhibits all the functional properties characterized by electrophysiological measurements in vivo. Consequently, the *Torpedo* AChR is ideally suited as a model membrane protein for the study of both lipid–protein interactions and general problems in reconstitution.

One of the important questions that has been addressed using the reconstituted receptor is how the physical and chemical characteristics of the membrane affect protein function (McNamee et al., 1986). It is known, for example, that the ability of the receptor to undergo both agonist-mediated allosteric-state transitions and channel gating is highly dependent on the lipid environment (Criado et al., 1984; Fong & McNamee, 1986, 1987; McNamee et al., 1986). Although such studies show that there is no absolute requirement for a particular class of lipids, it appears that both cholesterol and negatively charged lipids are important for successful reconstitution of ion-gating activity. Furthermore, it is known that perturbations in the membrane environment mediated by phospholipases (Andreasen et al., 1979), anesthetics (Taylor et al., 1983), and detergents (Heidmann et al., 1980) also affect receptor function.

One of the most powerful approaches for characterizing lipid–protein interactions involves the use of spectroscopic techniques which can differentiate between lipids at the lipid–protein interface and the bulk lipid population. Since the exchange rate constant of phospholipids is of the order of 10^7 s^{-1} at the interface of integral membrane proteins (Devaux & Seigneuret, 1985; East et al., 1985), appropriate techniques have included EPR and fluorescence with a time resolution of at least 10^{-7} s (Devaux & Seigneuret, 1985). The use of a variety of spin-labeled lipids has revealed the presence of two components in the EPR spectra of native (Marsh & Barrantes, 1978) and reconstituted (Ellena et al., 1983) membranes containing AChR. The motionally restricted component is absent from the EPR spectra of pure lipids and has been attributed to a lipid population which interacts with the protein.

It is not known what fraction of the number of lipids in contact with the receptor is actually required to support receptor function. Such information is particularly relevant when considering the effects of molecules such as detergents, which are expected to displace phospholipids during purification. In order to examine the role of lipids in stabilizing receptor, it is necessary to analyze receptor function over a range of lipid to protein ratios. Unfortunately, few methods for the controlled delipidation of membrane proteins have been reported. The most effective method is a centrifugation technique previously used to prepare complexes of $(Ca^{2+} + Mg^{2+})$-ATPase at defined lipid to protein ratios (Warren et al., 1974; East et al., 1985). Here, we describe the successful use of centrifugation methods to prepare AChR-containing membranes at a range of lipid to protein ratios.

We show that a minimum number of lipids are required to support the functional properties of the receptor. The data

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; ATPase, adenosinetriphosphatase; Carb, carbamylcholine; EDTA, ethylenediaminetetraacetic acid; DOPC, dioleoylphosphatidylcholine; EPR, electron paramagnetic resonance; ^{125}I-BTX, ^{125}I-monoiodinated α-bungarotoxin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AEM, alkaline-extracted membrane; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DPPC, dipalmitoylphosphatidylcholine; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; FTIR, Fourier-transform infrared.

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can be interpreted in terms of a model where the hydrophobic portions of functional receptor proteins are solvated by a single shell of lipids in accord with the notion of a lipid annulus. We also show that detergent inactivation of the acetylcholine receptor ion channel function depends on the *mole fraction* of detergent in the membrane and that the critical mole fraction of detergent is sufficient to deplete the annular lipids.

Materials and Methods

Preparation of Partially Pure Membranes Containing AChR. A crude membrane preparation partially enriched in AChR was prepared as follows. Frozen *Torpedo californica* electroplax tissue (600 g) was thawed, and 200-g portions were added to 200 mL of homogenization buffer [10 mM NaH_2PO_4, 5 mM EDTA, 5 mM EGTA, and 0.02% (w/v) NaN_3, pH 7.5] containing freshly added iodoacetamide (0.37 g) and PMSF (0.2 mL of a 200 mM fresh stock solution in ethanol). Each portion of tissue was homogenized for 2 min (4 × 30-s bursts) using a Polytron homogenizer (Brinkmann) at setting 7, and all steps were carried out at 0–4 °C. The combined homogenates were then centrifuged for 10 min at $5000g_{av}$ (Sorvall GSA rotor) and the supernatants filtered through four layers of cheesecloth. The pellets were resuspended in 240 mL of homogenization buffer containing freshly added iodoacetamide (0.44 g) and PMSF (0.24 mL) and homogenized for 60 s (4 × 15-s bursts), and the centrifugation step was repeated. The supernatant was then filtered through four layers of cheesecloth and the pellet discarded. The supernatants of both centrifugation steps were pooled and centrifuged at $34000g_{av}$ (Beckman type 19 rotor) for 125 min at 4 °C. The resulting supernatant was discarded and the pellet resuspended in 75 mL of buffer A [10 mM MOPS, 100 mM NaCl, 0.1 mM EDTA, and 0.02% (w/v) NaN_3, pH 7.4] at a protein concentration of 10–20 mg/mL. The crude membrane preparation was frozen and stored in liquid nitrogen until required. *Torpedo* electroplax tissue was obtained from Dr. H. Wang, University of California, Santa Cruz, CA.

Alkaline Extraction. For some experiments, native membranes were further enriched by sucrose density gradient centrifugation and alkaline extraction. Crude membranes corresponding to 200 g of electroplax tissue were suspended in 48 mL of 28% (w/v) sucrose in buffer A. The mixture was then layered in 8-mL aliquots onto discontinuous sucrose density gradients consisting of 10 mL of 30% (w/v), 12 mL of 35% (w/v), and 7 mL of 41% (w/v) sucrose in buffer A prepared in Beckman SW27 polyallomer tubes. The tubes were centrifuged at $95000g_{av}$ for 4 h at 4 °C (Beckman SW27 rotor) and the bands containing AChR collected and pooled. The membranes were subsequently washed by diluting 1:1 with buffer A and centrifuging at $95000g_{av}$ for 1 h at 4 °C. The pellets were combined and peripheral membrane proteins removed by alkaline extraction as described by Neubig et al. (1979). The alkaline-extracted membranes (AEMs) were frozen and stored in liquid nitrogen.

Preparation of (Carboxymethyl)choline Affinity Column. Affi-Gel 401 (Bio-Rad, Richmond, CA) (50-mL packed volume) was suspended in 50 mL of 40 mM DTT in 0.1 M Tris-HCl, pH 8.0, at room temperature in a 3 × 20 cm Econo column (Bio-Rad). The suspension was mixed by repeated inversion for 20 min and then allowed to pack. The gel was then washed with water to remove DTT followed by 100 mL of 50 mM sodium phosphate, pH 7.0. After being washed, the gel was resuspended, and 350 mg of bromoacetylcholine bromide (Damle et al., 1978) was added with vigorous mixing. The suspension was inverted occasionally during the 30-min reaction time and was then allowed to settle. The column was washed with 100 mL of water, and residual sulfhydryl residues were alkylated by adding 50 mg of iodoacetamide. After being washed with 200 mL of buffer A, the column was stored at 4 °C where it was found to be stable for 1–2 months. Derivatization of the gel was followed qualitatively by assaying for sulfhydryl groups using 300 μM DTNB in 0.1 M Tris-HCl at pH 8.0.

Purification and Reconstitution. Crude membranes (600 mg total protein) were diluted with buffer A to a protein concentration of 2 mg/mL and solubilized by adding solid sodium cholate, with gentle stirring, to a final detergent concentration of 1% (w/v). Stirring was continued for 30 min after which the mixture was centrifuged $95000g_{av}$ (Beckman type 35 rotor) for 1 h at 4 °C. The supernatants were filtered through four layers of cheesecloth, pooled, and applied to the affinity column (prepared above) at a flow rate of 2–3 mL/min.

For purification and reconstitution into asolectin (Applied Concentrates, Woodside, NY), the affinity column was washed with 100 mL of buffer B [1 mg/mL dioleoylphosphatidylcholine (DOPC) and 1% (w/v) cholate in buffer A]. The receptor was then eluted by applying 12 mL of buffer B containing 10 mM carbamylcholine (Carb) to the column. The column was then allowed to stand in the eluent for 30 min after which further elution with buffer B containing 10 mM Carb was resumed at a flow rate of 0.5 mL/min, and 2-mL fractions were collected. The protein concentration of the fractions was determined from the absorbance at 280 nm. An A_{280} value of 1.66 has been shown to correspond to a protein concentration of 1 mg/mL determined by the Lowry assay (Walker et al., 1982). The purified receptor was then reconstituted into asolectin vesicles at a lipid to protein molar ratio of 10000:1 by pooling the receptor-containing fractions to a protein concentration of 1–1.5 mg/mL and mixing the pooled receptor with a solution of 60 mg/mL asolectin and 4% (w/v) cholate in buffer A, previously sonicated to clarity under argon, at a ratio of 2:1 (v/v). The mixture was then dialyzed for 60 h at 4 °C against 4 L of buffer A with three buffer changes.

When purifying and reconstituting AChR into DOPC, a more elaborate washing procedure was employed to ensure complete exchange of receptor lipids for DOPC. After the column was loaded with the solubilized receptor solution, the column was washed with 100 mL of buffer B followed by 50 mL of buffer C [2.5 mg/mL DOPC and 1% (w/v) cholate in buffer A] and the column allowed to stand in buffer C for 3 h. Complete exchange of native lipids by DOPC was then achieved by washing the column with a further 50-mL portion of buffer C and allowing the column to stand overnight in the same buffer. The column was finally washed with 150 mL of buffer D [0.1 mg/mL DOPC and 0.5% (w/v) cholate in buffer A] and the receptor eluted as outlined above for asolectin membranes using buffer D containing 10 mM carbamylcholine as eluent. Reconstitution of receptor into DOPC at a lipid to protein molar ratio of 100:1 was achieved by pooling receptor-containing fractions to a protein concentration of about 1.0 mg/mL and dialyzing the mixture for 60 h at 4 °C against 4 L of buffer A with three buffer changes. Exchange of native lipids for DOPC was determined by gas–liquid chromatography of the fatty acid methyl esters according to Morrison and Smith (1964).

Concentration of Receptor Membranes. Most of the experiments used suspensions of AChR membranes at a concentration of 8 mg/mL, i.e., approximately 10-fold higher than that of the dialyzed membranes. The membranes were con-

centrated by centrifugation at 325000g_{av} for 2 h at 4 °C using a Beckman type SW 60 rotor. The membrane pellet was then resuspended to 10 mg/mL in buffer A using a small-volume homogenizer and the protein concentration determined. The concentrated membranes were frozen and stored in liquid nitrogen until required and then diluted to 8 mg/mL in buffer A prior to use.

Determination of Receptor Solubilization. The extent of AChR solubilization was determined from the protein and receptor concentrations in the supernatant after centrifugation. Briefly, 25 μL of AChR-containing membranes was mixed with an equal volume of cholate solution in buffer A to give a protein concentration of 4 mg/mL in the desired concentration of cholate. The mixture was incubated on ice for 2 h and then centrifuged in a Beckman Airfuge at 30 psi for 30 min. Following centrifugation, a small aliquot (0.5–2.0 μL) of the supernatant was diluted with buffer and assayed for either total protein or toxin binding sites.

Detergent inactivation profiles were determined by mixing the solubilized material together with the resuspended pellets and the concentrations of lipid and cholate adjusted to 20 mg/mL and 2% (w/v), respectively, using solutions of asolectin and cholate. The receptor was then reconstituted by dialyzing the mixture for 60 h at 4 °C against 4 L of buffer A with three buffer changes.

Sucrose Density Gradient Centrifugation. The association of lipid and protein under different conditions of solubilization was determined by centrifugation on continuous sucrose density gradients. Typically, membranes containing 1 mg of protein and the desired amount of cholate in buffer A (total volume 0.5 mL) were incubated on ice for 1–2 h and layered onto continuous sucrose density gradients [20–60% (w/v) in buffer A] prepared in Beckman SW60 cellulose nitrate tubes (4.2-mL volume). The tubes were centrifuged at 325000g_{av} for 22 h at 4 °C (Beckman SW60 rotor). Fractions of 100 μL were collected manually.

The distribution of receptor was determined by incubating receptor-containing membranes with a trace of ^{125}I-α-bungarotoxin (^{125}I-α-BTX; ~0.5 pmol/mg of AChR) for 2 h at room temperature prior to solubilization.

The distribution of membrane lipids was assessed with [^{14}C]dipalmitoylphosphatidylcholine ([^{14}C]DPPC) incorporated into the membranes as follows: receptor-containing membranes (1 mL) at a protein concentration of 10 mg/mL were pelleted by centrifugation at 325000g_{av} for 2 h. The resulting receptor-free supernatant was removed and added to a thin film of [^{14}C]DPPC (1×10^{-8} mol) prepared by drying down 100 μL of [^{14}C]DPPC (10 μCi/mL in toluene) with a stream of argon. Solid sodium cholate (20 mg) was added to the mixture which was then sonicated for 5 min above the phase transition temperature of the lipid (>40 °C), cooled on ice, and recombined with the membrane pellet. The pellet was solubilized by gentle resuspension and reconstituted by dialyzing the mixture for 60 h against 4 L of buffer A with three changes. The [^{14}C]DPPC-labeled membranes were frozen and stored in liquid nitrogen.

The distribution of cholate was monitored by incorporating [^{3}H]cholate into the stock cholate solution to a specific activity of 32 nCi/mg of total cholate.

Preparation of AChR Membranes at Different Lipid to Protein Ratios. For the preparation of membranes at differing lipid to protein ratios, samples containing 1 mg of AChR were incubated on ice with the desired amount of cholate in 500 μL of buffer A for 2 h with occasional mixing. The mixture was layered onto discontinuous sucrose gradients of 3.6 mL of 25% (m/v) sucrose in buffer A with a 600-μL "cushion" of 60% (w/v) sucrose in buffer A and centrifuged overnight at 325000g_{av} at 4 °C. The opalescent bands obtained at the 25–60% interface were collected, analyzed for lipid and protein content, and reconstituted back into asolectin as described below.

Reconstitution of AChR Back into Asolectin Vesicles. In some instances, it was necessary to test for irreversible losses of ion channel activity. Membranes were reconstituted back into asolectin by adjusting the levels of lipid (asolectin) and cholate to 20 mg/mL and 2% (w/v), respectively. The solubilized mixture was incubated on ice for 2 h with occasional mixing and then dialyzed at 4 °C for 60 h against 4 L of buffer A with three buffer changes.

Assays for AChR Function. Acetylcholine receptor concentrations were determined by measuring equilibrium binding of ^{125}I-α-BTX as outlined by Walker et al. (1981). The orientation of receptor toxin binding sites in reconstituted membranes was determined by comparing equilibrium toxin binding in the presence and absence of the assay detergent.

Ion flux into reconstituted membranes was determined by manually mixing 50–100 μL of membranes (0.2–1 mg/mL AChR in buffer A) with 15 μL of $^{86}Rb^{+}$ in buffer A with, or without, 10 mM Carb. After 30 s, the reaction mixture was applied to a 2-mL disposable column (QSY, Isolab, Inc. Akron, OH) containing Dowex 50W-X8 [prepared according to Epstein and Racker (1978)] and the column washed with 3 mL of ice-cold 175 mM sucrose in water. Vesicles containing trapped $^{86}Rb^{+}$ were collected and counted as described previously (Walker et al., 1982; Jones et al., 1987a). The internal volume of the vesicles was determined by incubating the membranes for 2 days with $^{86}Rb^{+}$ in buffer A containing 10 mM Carb before filtering. All flux assays were done in triplicate.

The ability of the receptor to undergo affinity transitions characteristic of the desensitization process was determined by a toxin rate binding assay. Briefly, receptor-containing membranes (5 nM toxin sites) were mixed with 30 nM fresh ^{125}I-α-BTX in a total volume of 700 μL of buffer A containing 0.5 mg/mL BSA. Toxin binding to receptor was determined by filtering 100-μL aliquots at 30-s intervals as described by Walker et al. (1982). A pseudo-first-order rate constant for the toxin–receptor interaction was determined by regression analysis of the linearized form of the rate equation. Rate constants for toxin binding to the high- and low-affinity forms of the receptor were determined from the rate of toxin binding to receptor samples preincubated or coincubated with 5 μM Carb, respectively.

Electron Microscopy. Membrane samples were prepared for negative staining by applying 15 μL of sample (0.2 mg/mL in buffer A) to parlodian-coated copper grids rendered hydrophilic by glow discharging. The grids were washed with a small volume (usually five drops) of phosphate-free buffer A and stained with 1% (w/v) uranyl acetate at pH 5.5. The samples were viewed at 100 kHz with a Philips EM 400 electron microscope fitted with an anticontamination trap.

Other Assays. Lipids were extracted from the membrane samples according to the method of Bligh and Dyer (1959), and the phospholipid concentration was determined according to the method of Yoshida et al. (1980). Phospholipid purity was determined by two-dimensional thin-layer chromatography (Bakerflex plates, silicic acid + fluorophore) using a solvent system of $CHCl_3$–CH_3OH–H_2O–CH_3COOH (65:25:4:1 by volume) in the first dimension and $CHCl_3$–CH_3OH–H_2O–NH_3 (65:25:4:1 by volume) in the second dimension (Siakatos

& Rouser, 1965). Lipid spots were visualized with I_2 vapor and phospholipids with the modified Dittmer–Lester stain (Ryu & MacCoss, 1979). Protein was determined according to Lowry et al. (1951) using fat-free bovine serum albumin (Sigma, fraction V) as standard. For samples with a high lipid content, the Lowry procedure was performed in the presence of 2% (w/v) SDS to clarify the solutions. Protein purity was determined by electrophoresis on 7.5% (w/v) SDS–polyacrylamide slab gels according to Ames (1974).

Radioactivity was counted with a Packard γ scintillation spectrometer for samples containing ^{125}I. Samples containing [^{14}C]DPPC or [^{3}H]cholate were first mixed with PCS (Amersham)/xylene (1:1) scintillation fluid and counted with a Beckman scintillation counter. Samples containing $^{86}Rb^+$ were similarly counted, but in the absence of fluor.

Results

Purification and Reconstitution of AChR Membranes. In this study, three different types of membrane preparation from *Torpedo californica* electroplax were employed. All three receptor preparations have distinctive features, and an understanding of their properties is a central element of this study. The first preparation was alkaline-extracted native membranes (AEMs) purified by differential and sucrose density gradient centrifugation, followed by brief exposure to alkali pH. Alkaline extraction has been shown to release most of the peripheral proteins while having little effect on the functional properties of the receptor (Neubig et al., 1979). The AEMs therefore serve as a model for the receptor in its native lipid environment. Assuming an average molecular weight for the protein of 250 000, a lipid to protein molar ratio of 400:1 (Φ = 400) can be estimated for the AEMs.

In addition, two types of reconstituted membranes were prepared by affinity chromatography of cholate-solubilized crude membranes and subsequent reconstitution according to the cholate dialysis procedure of Epstein and Racker (1978). The first type of membrane was AChR reconstituted into asolectin (aso–AChR) at Φ = 10 000. The asolectin-reconstituted membranes support agonist-mediated ion translocation and have been extensively characterized. They therefore serve as an excellent model with which to study the functional properties of the AChR (Anholt et al., 1981, 1982; Huganir & Racker, 1982). AChR was also reconstituted into the synthetic lipid dioleoylphosphatidylcholine (DOPC–AChR) at Φ = 100. DOPC–AChR was chosen as an example of the purified receptor in a defined lipid environment.

The AEMs had a specific ^{125}I-α-BTX binding activity of 1–2 nmol/mg of total protein, indicating that about 25% of the total protein was AChR. The heterogeneous protein composition of the AEMs was revealed by SDS–polyacrylamide gel electrophoresis and showed the prescence of several other contaminants, notably a band of high molecular weight (100 000) probably corresponding to ($Na^+ + K^+$)-ATPase. In contrast, both asolectin- and DOPC-reconstituted membranes had a specific ^{125}I-α-BTX binding activity of 6–9 nmol/mg of total protein, consistent with a value of 8 nmol/mg expected for pure AChR. Analysis of the protein composition by SDS–polyacrylamide gel electrophoresis revealed only the presence of bands corresponding to the four types of receptor subunit.

The ability of the receptor preparations to undergo ligand gating of the receptor channel was assessed by using the manual ion flux assay outlined previously (Walker et al., 1982). As shown in Table I, the three types of membranes differed markedly in their functional properties. The flux activity of the native membranes was relatively low, as previously observed for alkaline-extracted membranes. Aso–AChR showed full control of the channel gating properties and gave the highest flux activities of the three membrane preparations tested. In contrast, DOPC–AChR was unable to undergo either the allosteric transitions characteristic of desensitization or the agonist-mediated changes in ion permeability (Fong & McNamee, 1986). DOPC–AChR at Φ = 100 therefore showed none of the characteristics of a functional receptor. The low lipid sample does not form sealed vesicles, so the lack of impermeability response was expected (Earnest et al., 1987). The allosteric transition can be detected at low lipid to protein ratios provided the proper lipid environment is present (Fong & McNamee, 1986).

Table I: Properties of AChR-Containing Membranes[a]

membranes	Φ[b]	^{125}I-BTX binding (pmol/mg of protein)[c]	ion flux activity[d] (μM^{-1})
(A) Membranes Prior to Re-reconstitution			
alkaline-extracted native membranes	400	1.23 (2)	0.5
aso–AChR	10000	6.88 ± 0.15 (3)	4.8 ± 2.5 (9)
DOPC–AChR-1	100	7.68 ± 1.03 (5)	
DOPC–AChR-2	37		
(B) Membranes after Reconstitution into Asolectin			
alkaline-extracted native membranes	10000	nd[e]	2.3 (2)
aso–AChR	10000	7.63 ± 1.01 (5)	4.3 ± 1.0 (3)
DOPC–AChR-1	10000	6.08 ± 1.76 (3)	5.3 ± 1.4 (3)
DOPC–AChR-2	10000	7.06 ± 1.72 (5)	1.9 ± 1.0 (3)

[a] Four membrane preparations were analyzed for ^{125}I-α-BTX binding and for carbamylcholine-induced $^{86}Rb^+$ uptake. Methods for purifying membranes and/or AChR are given under Materials and Methods. [b] Φ represents the lipid to protein molar ratios of the membranes. [c] ^{125}I-α-BTX binding expressed in picomoles of toxin sites per milligram of protein ± standard deviation. [d] $^{86}Rb^+$ ion flux activity expressed as {[cmp(+Carb) − cpm(−Carb)]/cpm(total)}[AchR]. cpm(total) represents the total internal volume of the vesicles measured after 48-h incubation with $^{86}Rb^+$. [e] Not determined.

The functional integrity of the receptor protein itself could be readily demonstrated by mixing alkaline-extracted or -reconstituted membranes with an excess of asolectin in cholate and dialyzing. Membranes which were reconstituted back into asolectin all showed the ability to undergo agonist-induced ion translocation similar to that found for pure aso–AChR (Table I). The ability to be able to reconstitute the AChR back into asolectin provides a powerful means for detecting any irreversible loss of receptor function.

Solubilization of AChR. The extent to which cholate could extract AChR from the membrane was found to be highly dependent on the type of membrane preparation used (Figure 1). Since the protein concentration was identical for all samples, the extent of solubilization depended primarily on the total lipid concentration. The concentration of cholate required to solubilize aso–AChR was considerably higher than that required to solubilize either DOPC–AChR or AEMs. The cholate concentrations for half-maximal solubilization were 0.6%, 0.8%, and 7% (w/v) for DOPC–AChR, AEMs, and aso–AChR, respectively. Only 70–80% of the receptor was extracted from the AEMs, whereas all the receptor was extracted from the purified, reconstituted membranes.

Detergent Inactivation of AChR. A similar dependence on the type of membrane preparation employed was found for the irreversible inactivation of AChR ion channel activity. Receptor-containing membranes were incubated at various concentrations of detergent and then incorporated into asolectin vesicles by reconstitution under optimal conditions. For each

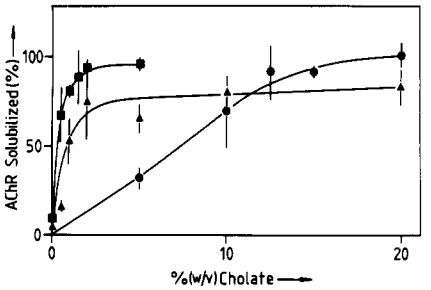

FIGURE 1: Comparison of solubilization of AChR from native and reconstituted membranes. Receptor-containing membranes, at a protein concentration of 4 mg/mL, were suspended at 4 °C for 2 h in buffer A at the cholate concentrations shown in a final volume of 50 μL. The mixtures were then centrifuged for 30 min in a Beckman airfuge and the supernatants assayed for ^{125}I-α-BTX binding activity. DOPC–AChR (Φ = 100) (■); alkaline-extracted native membranes (Φ = 400) (▲); aso–AChR (Φ = 10000) (●). Numbers in parentheses (Φ) are the lipid to protein molar ratios of the starting membranes. Values are shown as the mean ± the standard deviation of at least three measurements.

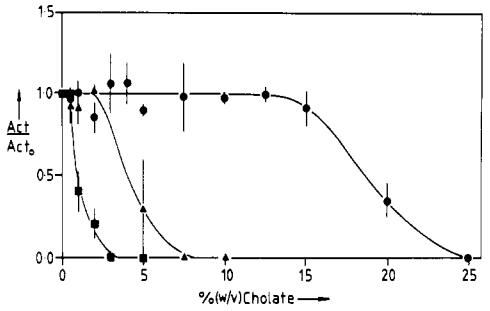

FIGURE 2: Irreversible inactivation of receptor ion channel function by cholate. Receptor-containing membranes were suspended at a concentration of 4 mg/mL in buffer A supplemented with the indicated concentration of cholate and stored on ice for 2 h with occasional mixing. The mixtures were then reconstituted back into asolectin vesicles by adding asolectin and cholate to give a final concentration of 20 mg/mL lipid and 2% (w/v) cholate and dialyzed for 3 days against 4 L of buffer A with three changes. The reconstituted membranes were then tested for agonist-induced $^{86}Rb^+$ influx as described under Materials and Methods. DOPC–AChR (Φ = 100) (■); alkaline-extracted membranes (Φ = 400) (▲); aso–AChR (Φ = 10000) (●). Values are shown as means ± the standard deviations for three sets of samples assayed in triplicate.

type of membrane, there was a strong irreversible inhibition of ion channel activity above a threshold concentration of cholate. The concentration of cholate at which the onset of inactivation occurred was lowest for DOPC–AChR and highest for aso–AChR and also appeared to correlate well with the lipid content of the membranes.

Sucrose Gradient Analysis. The relationship between the cholate concentration and the lipid content of the receptor membranes was further examined by sucrose density gradient centrifugation using a technique adapted from Warren et al. (1974). Membranes were treated with varying concentrations of cholate and centrifuged to equilibrium on continuous sucrose gradients. The distribution profiles of AChR and lipid are shown in Figure 3. A single symmetrical peak of toxin binding activity indicated a population of receptor-containing membranes of homogeneous density. In no instance were pellets of toxin binding material detected at the bottom of the tubes; aggregation of receptors had clearly not occurred, even at the highest cholate concentrations. In the absence of AChR, pure ^{125}I-α-BTX was distributed at the top of the gradients (data not shown). Figure 3 also shows that in the absence of detergent all the lipid comigrates with the receptor protein (Figure 3C). Liposomes prepared in the absence of AChR

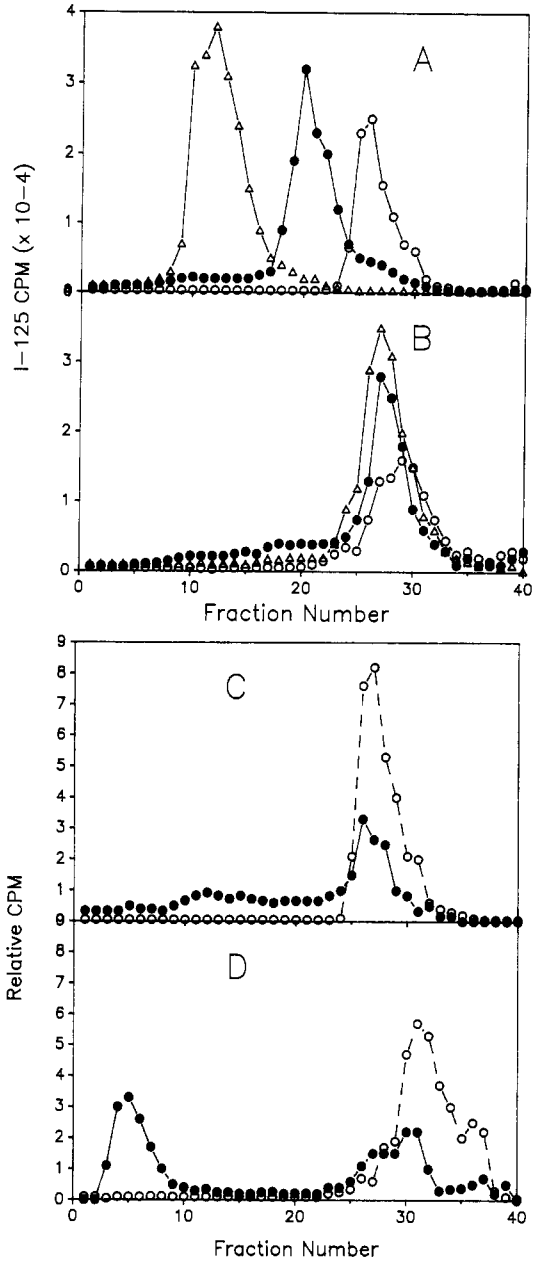

FIGURE 3: Isopycnic sucrose gradient centrifugation of receptor-containing membranes solubilized at different concentrations of cholate. Membranes, trace-labeled with [^{14}C]DPPC or ^{125}I-α-BTX, were suspended at a protein concentration of 4 mg/mL in buffer A supplemented with the indicated concentration of cholate, layered onto cholate-free, 0–60% (w/v) continuous sucrose density gradients, and centrifuged as described under Materials and Methods. The locations of receptor and lipid components were determined from the distribution of ^{125}I-α-BTX or [^{14}C]DPPC in membranes trace-labeled as described under Materials and Methods. (A) Distribution of AChR-containing membranes as detected by ^{125}I-BTX binding for the three different preparations used: alkaline-extracted membranes (●); DOPC–AChR membranes at a lipid to protein mole ratio (Φ) of 100 (○); AChR reconstituted into asolectin at a high lipid to protein ratio of Φ = 10000 (△). (B) Distribution of AChR after solubilization of membranes in cholate and centrifugation through a detergent-free gradient. A concentration of cholate was selected for each membrane to give similar sedimentation profiles: alkaline-extracted membranes at 2.5% (w/v) cholate (●); DOPC–AChR membranes at 1.5% (w/v) cholate (○); asolectin–AChR membranes at 20% (w/v) cholate (△). (C) Distribution of both [^{14}C]DPPC and ^{125}I-BTX binding for DOPC–AChR membranes (Φ = 100) in the absence of cholate. [^{14}C]DPPC cpm (●) and ^{125}I-BTX cpm (○) plotted on same scale. Actual peak value for ^{14}C was 320 and for ^{125}I was 27 300. Each labeled sample was run on a separate gradient. (D) An example of the distribution of [^{14}C]DPPC and ^{125}I-BGT after treating DOPC membranes with cholate prior to gradient centrifugation. [^{14}C]DPPC (●) and ^{125}I-BTX (○). Note that some of the lipid remains at the top of the gradient and some remains associated with the AChR, which moves to a higher density. Cholate concentration was 1% (w/v).

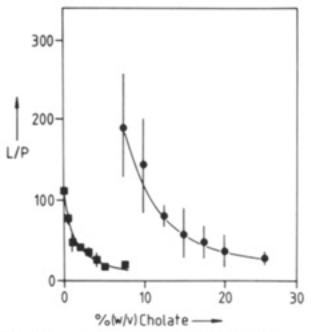

FIGURE 4: Delipidation of receptor-containing membranes by increasing the cholate concentration. Reconstituted membranes were incubated at a protein concentration of 4 mg/mL in buffer A supplemented with cholate at the concentrations indicated. The mixtures were then centrifuged on discontinuous 25–70% (w/v) sucrose gradients as described under Materials and Methods. The lipid to protein molar ratio (Φ) of the resulting bands was then determined. Values are shown as the means ± the standard deviations of at least three measurements. DOPC–AChR (■); aso–AChR (●).

were located near the top of the gradients as found by Anholt et al. (1981). The distribution of AChR was dependent on the lipid content of the membranes employed, those membranes having the highest lipid content distributed at lower sucrose densities than membranes with lower lipid content (Figure 3A). In the presence of cholate, the lipid was distributed into two populations: lipid retained at the top of the gradient and lipid associated with the receptor (Figure 3D). When the concentration of cholate used to solubilize the AChR was increased, a greater fraction of lipid was retained at the top of the gradient, while the fraction of lipid associated with the receptor decreased. The effect of cholate was therefore to deplete the membranes of lipid to an extent which increased with the concentration of cholate used in the solubilization step. Depletion of lipids from the membrane was accompanied by a shift in the AChR distribution profile to a lower sedimentation equilibrium position. Significantly higher concentrations of cholate were required to achieve comparable distributions of AChR when the lipid content of the starting membrane preparations was raised (Figure 3D). Even at the highest concentrations of cholate employed, the level of cholate associated with the receptor was negligible. All the cholate remained at the top of the gradients based on trace labeling with [^{3}H]cholate (data not shown). At all detergent concentrations examined, we found no evidence for dissociation of receptor subunits nor was there any reduction in the ability of the receptor to bind ^{125}I-α-BTX.

Preparation of AChR Complexes at Different Lipid to Protein Ratios. To further examine the relationship between the detergent inactivation profiles and the levels of lipid associated with the AChR, the receptor-containing membranes were prepared at various lipid to protein ratios using a discontinuous sucrose density gradient technique. The relationship between the lipid to protein ratio of the membranes and the cholate concentration is shown in Figure 4. In accord with the results from the continuous gradients, it was clear that by solubilizing the membranes with an increased amount of cholate the lipid content of the membranes could be reduced. To achieve a particular lipid to protein ratio after centrifugation, aso–AChR required a greater concentration of cholate in the solubilization mixture than did DOPC–AChR. Interestingly, not all the lipid could be displaced from the protein. Even at the highest concentrations of cholate, a residual number of around 20 lipids remained associated with the receptor.

Electron Microscopy. It was possible to study the morphology of the receptor-containing membranes by electron microscopy of negatively stained samples. The results for DOPC–AChR are shown in Figure 5 for samples prepared at Φ = 100, 45, and 20. The gross morphology of such complexes indicates that the membranes form bilayer sheets with no evidence for a population of membrane vesicles. In fact, at these low lipid to protein ratios, there was little contamination by either free receptors or lipid vesicles devoid of receptor, which are often observed in preparations made at higher lipid to protein ratios. From a more detailed study of the morphology, the AChR proteins appeared as characteristic doughnut-shaped particles of around 80-Å diameter and were observed only in membrane preparations that contained receptor proteins. The dense packing of the AChR at low lipid to protein ratios made it difficult to determine if AChRs in

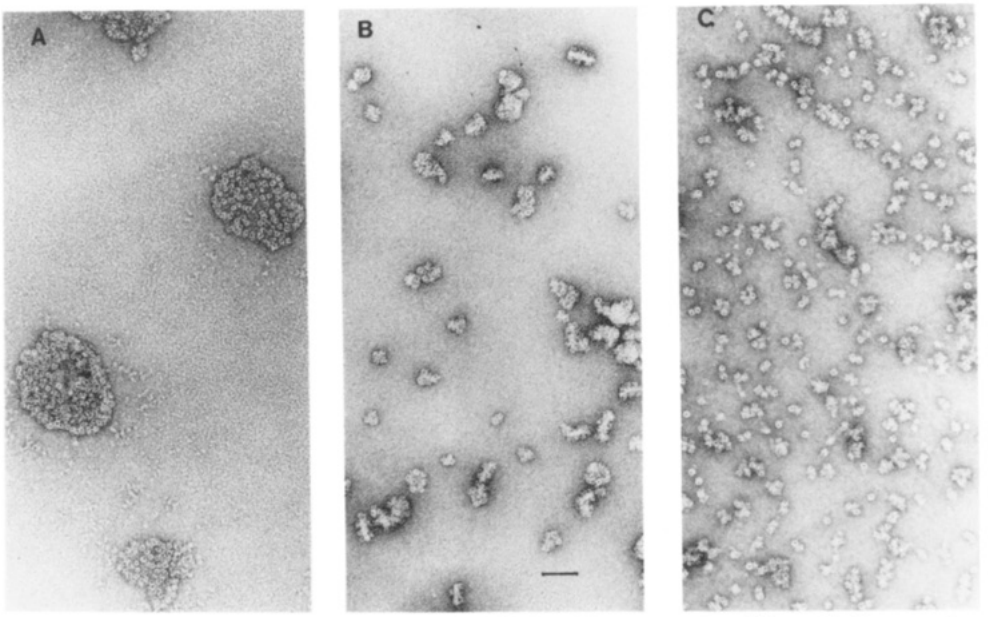

FIGURE 5: Electron micrographs of negatively stained reconstituted DOPC–AChR prepared at different lipid to protein molar ratios by the centrifugation method. (A) DOPC–AChR (Φ = 100) starting material. (B) DOPC–AChR (Φ = 45), 1% cholate. (C) DOPC–AChR (Φ = 20), 7.5% cholate. AChR appears as doughnut-shaped particles of radius 80 Å. Magnification 93600×. Note the increase in the packing of the particles as the lipid content of the membranes is reduced.

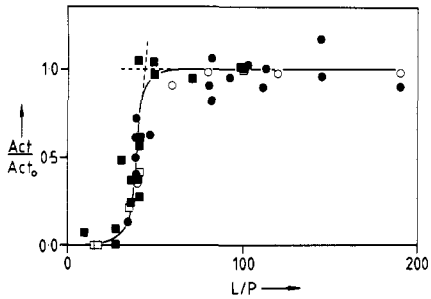

FIGURE 6: Dependence of receptor ion channel function on the lipid to protein molar ratio (Φ). AChR at a protein concentration of 4 mg/mL was incubated with various concentrations of cholate as described in the legend to Figure 4. The mixutres were centrifuged on discontinuous sucrose gradients and the resulting complexes analyzed for lipid:protein molar ratio and irreversible ion channel activity at 4 °C. DOPC–AChR (■); aso–AChR (●). Data are also shown for DOPC–AChR (□) and aso–AChR (○) from Figure 2 assuming that detergent-induced inactivation results from displacement of receptor lipids. In this instance, the lipid to protein molar ratios corresponding to the concentrations of cholate causing inactivation in Figure 2 were determined from Figure 4.

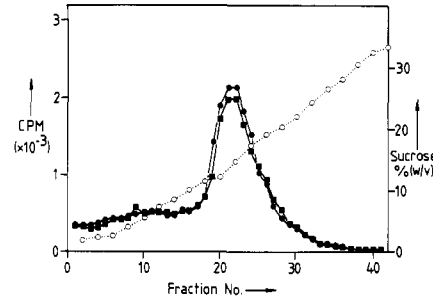

FIGURE 7: Sucrose gradient centrifugation analysis of receptors reconstituted into asolectin vesicles before and after delipidation. Aso–AChR delipidated to a lipid to protein molar ratio of 34:1 by the centrifugation method and then reconstituted at a Φ value of 10000 (■). Aso–AChR (Φ = 10000) starting material centrifuged in the absence of cholate and then analyzed by sucrose gradient centrifugation (●). Distribution of sucrose determined by the refractive index (○).

these membranes were in the form of monomers or dimers. A comparison of the micrographs of membranes prepared at different lipid to protein ratios showed that as the lipid content of the membranes was lowered there was an increase in the packing density of the receptors, confirming the evidence of the sucrose gradients.

Delipidation and AChR Function. An important feature of the centrifugation method is that receptor-containing membranes could be prepared at defined lipid to protein ratios. By isolating the complexes, it was therefore possible to study the relationship between the lipid content of the membranes and AChR function. Two aspects of receptor function were studied: the ability of the receptor to bind agonists and allow ion flux, and the ability of the receptor to undergo allosteric transitions characteristic of the desensitization process.

The assay of AChR channel activity depends on the ability to measure translocation of ions into an appropriate compartment, usually sealed vesicles. Since the membranes prepared at low lipid to protein ratios do not form sealed vesicles (Figure 5), it was necessary to reconstitute the AChR into vesicular form. This was achieved by mixing the AChR-containing complexes with excess asolectin and cholate and reconstituting by dialysis. The activity of the ion channels for the purified AChR, following reconstitution of AChR back into asolectin, is shown as a function of the lipid to protein mole ratio measured after solubilization with different amounts of cholate and centrifugation into cholate-free sucrose gradients (Figure 6). It is clear that irreversible inactivation of the AChR ion channels is not linearly related to the lipid content of the membranes but rather there is a threshold lipid to protein molar ratio below which the channel activity decreases significantly. For all three types of membrane preparations, the minimum number of lipids required to protect receptor channel function was independent of either the lipid content or the composition of the initial membrane preparation used. At least 45 mol of lipid per mole of AChR was required to maintain full channel function. Complete channel inactivation occurred when Φ was less than 20.

To rule out the possibility that delipidated AChR might remain functional but fail to be incorporated properly into vesicles, the membranes obtained by re-reconstituting highly delipidated membranes into asolectin were compared with asolectin vesicles containing AChR which had not been delipidated. As shown in Table I, there were no significant differences in the specific toxin binding activity of the AChR.

In addition, the internal volumes of the membranes were identical and characteristic of fully sealed vesicles. Loss of flux activity could also have arisen from lack of incorporation of AChR into the asolectin vesicles, a particularly strong possibility since delipidation could affect those regions required to anchor the protein in the bilayer. To test for incorporation, sucrose density gradients were used to compare the distribution profiles of AChR, isolated at a lipid to protein molar ratio of 34:1, and re-reconstituted into asolectin, with aso–AChR which had been centrifuged without cholate (and therefore not delipidated). The results in Figure 7 show that the distribution of AChR was identical in both cases. No toxin binding material was detected at the bottom of the gradients, indicating that all the AChR had been incorporated into lipid vesicles. The loss of channel activity, shown in Figures 2 and 6, using the re-reconstitution protocol, can therefore be attributed to a direct effect on the receptor protein.

Lipid Depletion and Allosteric Transitions. In the prolonged presence of agonists, the AChR undergoes an allosteric transition from a state of low affinity to one of high affinity for agonists. The affinity-state transition is characteristic of the desensitization process and is sensitive to the lipid environment of the receptor (Fong & McNamee, 1986). The ability of the receptor to undergo allosteric transitions was determined by a competition assay where the pseudo-first-order rate constant for association of ^{125}I-α-BTX with AChR was measured in the presence or absence of agonist. Unlike the ion flux assays, the toxin rate binding method does not require sealed vesicles and is particularly useful for samples prepared at low lipid to protein ratios. The effect of changing the lipid to protein molar ratio on the allosteric transitions in aso–AChR is shown in Figure 8A,B. Data for AChR reconstituted into DOPC were not available since AChR does not undergo the allosteric-state transitions in pure DOPC (Fong & McNamee, 1986).

For membranes reconstituted at high Φ values, the presence of agonist decreased the rate at which toxin associates with the receptor in a dose-dependent manner. In this study, a Carb concentration of 5 μM was used so that rate of toxin association to the low- and high-affinity states of the receptor could be easily resolved. Under these conditions, preincubation with agonist shifted the AChR into a high-affinity state and caused a marked depression of the toxin binding rate. In contrast, coincubation of the AChR gave an intermediate rate corresponding to toxin binding to the low-affinity AChR state. For aso–AChR at low lipid to protein ratios, the rate of toxin binding to AChR following preincubation with Carb was not greatly different from that for the binding of toxin to the AChR coincubated with Carb. Receptors in the delipidated

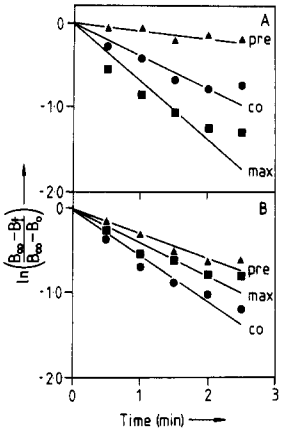

FIGURE 8: Effect of delipidation on the allosteric-state transitions of aso–AChR stabilization in a low-affinity state. The initial rate of toxin binding was determined for receptor-containing membranes (70 nM toxin binding sites) mixed with ^{125}I-α-BTX (■) or ^{125}I-α-BTX coincubated with 5 μM carbamylcholine (●) or for membranes incubated with 5 μM Carb prior to addition of toxin (▲). (A) Aso–AChR at a Φ value of 10 000. (B) Aso–AChR at a low Φ value of 34.

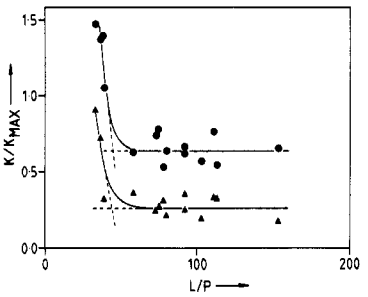

FIGURE 9: Dependence of the allosteric-state transitions on the lipid to protein molar ratio for aso–AChR membranes. Pseudo-first-order rate constants for the low- and high-affinity receptor states (k_{co} and k_{pre}, respectively) were determined and expressed relative to the rate constant for the association of toxin with AChR in the absence of Carb, k_{max}. Aso–AChR, k_{co}/k_{max} (●); aso–AChR, k_{pre}/k_{max} (▲).

membranes therefore appeared to exist in a state of low affinity for agonists. The rate constants corresponding to the maximum rate of toxin binding obtained in the absence of agonist and those obtained on co- or preincubation with agonist were designated k_{max}, k_{co}, and k_{pre}, respectively. By plotting the ratio of k/k_{max} for membranes either coincubated or preincubated with agonist, the effect of changing the lipid to protein ratio on agonist-induced affinity-state transitions was even more apparent (Figure 9). Clearly, loss of the low- and high-affinity transition induced by agonists only occurred below a minimum number of lipids. The number of lipids required to fully support the affinity-state transitions was around 45 per AChR, identical with that required to protect AChR ion channel function.

Discussion

From the results presented here, we suggest that a minimum number of lipids is required to protect the functional properties of the acetylcholine receptor. We have used a novel centrifugation method to reconstitute AChR as membrane complexes over a range of defined lipid to protein molar ratios. Our results show that when the lipid to protein molar ratio of the membrane is reduced below 45:1 the receptor is stabilized in a low-affinity form for agonist binding, and the ion channels are irreversibly inactivated. Displacement of lipids from the receptor appears to be an integral feature in the detergent-induced inactivation of the receptor.

Solubilization and Inactivation Depend on the Mole Fraction of Detergent in the Membrane. A central feature of our results is the extent to which the effects of the detergent cholate on receptor solubilization and activity differ among the membrane preparations employed. Since the protein concentration was identical in all the experiments, we have attributed such differences to the lipid content of the membranes. Both solubilization and inactivation of receptor by cholate were a function of the *mole fraction* of detergent in the membrane rather than the total concentration of cholate used in the solubilization mixture.

Our results are consistent with the solubilization scheme proposed by Reynolds (1982), in which detergent initially binds to the membrane and then at a critical detergent mole fraction fragmentation and mixed micelle formation occurs.

The correlation between the mole fraction of detergent in the membrane and disruption of receptor function provides several possible mechanisms for inactivation. For example, cholate has been shown to act like a local anesthetic at low concentrations (0.007% w/v) both in vivo (Briley & Changeux, 1978) and in vitro (Heidmann et al., 1978). Such an explanation for the effect of cholate is unappealing since the anesthetic should be reversible (Heidmann et al., 1978; Anholt et al., 1981). Also, re-reconstitution was performed at identical lipid and detergent concentrations for active and inactive samples, so the irreversible inactivation described here cannot readily be ascribed to low levels of residual detergent. It is possible that inactivation might result from loss of an essential lipid component. However, this also seems highly unlikely since we observed progressive irreversible receptor inactivation for DOPC–AChR where there is only one lipid component present prior to delipidation. Furthermore, inactivation by loss of some essential lipid factor might be expected to be reversible on reconstitution into a heterogeneous lipid mixture, such as asolectin. Since the concentration of detergent required to inactivate the receptor is higher than that necessary for maximal solubilization, it seems more probable that at inactivating concentrations the detergent causes extensive displacement of lipids from the receptor.

Delipidation by a Centrifugation Technique. An important advantage of the centrifugation method used in the studies reported here is the ease with which membranes can be reproducibly prepared at defined lipid to protein ratios simply by increasing the concentration of detergent. Other methods for delipidating the receptor, such as extensive washing with detergents (Chang & Bock, 1979) or extraction with organic solvents, are notoriously difficult to control. An interesting aspect of all the results is the difficulty in removing the last 20 lipids from the receptor. A similar result has also been found when delipidating the (Ca^{2+} + Mg^{2+})-ATPase using the centrifugation method and provides good evidence for a strong interaction between lipids and integral membrane proteins (East et al., 1985). Nevertheless, there is no evidence in the case of AChR for a strong interaction with particular lipids of the type suggested for cytochrome oxidase (Robinson et al., 1980). For example, it is possible to completely exchange receptor-associated lipids for test lipids with the column equilibration method described here, and in agreement with Chang and Bock (1979), we found no evidence for enrichment of specific lipids on delipidation.

A Minimum Number of Lipids Are Required for Receptor Function. The effects of lipid content on receptor function were tested by assessing irreversible inactivation of channel activity and by analyzing the allosteric transitions in ligand binding thought to accompany the desensitization process.

Results from both assays demonstrate that a minimum number of lipids are required to protect receptor ion channel activity. However, it is not possible to determine whether increasing cholate concentrations cause inactivation of the receptors by progressive denaturation of the entire channel population or by an increase in the fraction of receptors containing inactive channels. This question could be resolved by detailed analysis of ion channel activity in terms of either channel conductances or lifetimes using single-channel recording techniques (Labarca et al., 1985). Analysis of the sucrose gradient profiles and electron micrographs suggests that a single homogeneous receptor population exists at all cholate concentrations tested, indicating that the structural differences between active and inactive channels do not represent gross distortions of the protein or the membrane. It appears that the *ion channel* activity is much more sensitive to perturbations in the lipid environment than any other functional property of the receptor. For example, several groups have shown that many lipids which support allosteric transitions in the AChR do not support functional ion channels (Criado et al., 1982, 1984; Fong & McNamee, 1986). In no instance have lipids which support ion channel activity without the allosteric transition been reported.

The effect of delipidation on the ability of the receptor to undergo agonist-induced allosteric transitions paralleled the loss of ion channel activity. Below a lipid to protein molar ratio of 45:1, the receptor was stabilized in a state of low affinity for agonist. Our results agree well with those of Chang and Bock (1979), who used native membranes.

Minimum Number of Lipids Required To Support Receptor Function Is Consistent with a Lipid Annulus. From the activity measurements, it is clear that the minimum number of lipids required to support receptor function ($N_{act.}$) is around 45, irrespective of the lipid to protein ratio or the chemical composition of the starting membrane preparations. The value of N can be compared with that derived by other methods. The most direct method is to calculate the minimum number of lipids required to completely surround the perimeter of the receptor from simple geometry. An important consideration in such calculations is the value for the diameter of the intramembranous portions of the receptor, particularly since there is good evidence that the relevant dimensions are somewhat smaller than those directly visualized by negative-stain electron microscopy (Brisson & Unwin, 1985; Popot & Changeux, 1984). By use of the dimensions of Wise et al. (1979), a value of 45 for the number of lipids at the lipid–protein interface has been calculated (Ellena et al., 1983). A similar value for the number of lipids in contact with the receptor has been estimated from the motionally perturbed components in the EPR spectra of spin-labeled reconstituted membranes (Ellena et al., 1983) using the formalism of Brotherus et al. (1981). The close agreement between the number of perturbed lipids and the number of lipids predicted to surround the intramembranous portion of the receptor was taken as evidence that the protein-perturbed component originates from the first shell of lipids around the protein.

The agreement among the values of N determined by EPR, functional activity, and geometric measurements provides good evidence that the same population of lipids is involved. Consequently, the simplest interpretation of the available data is that the AChR is surrounded by a "shell" of 45 lipids, which is required for receptor function.

Detergents Inactivate the Receptor during Solubilization by Displacing Annular Lipids. On the basis of the results, we propose that detergents inactivate receptor ion channels and stabilize the receptor in a state of low-affinity agonist binding by displacing annular lipids. The most convincing evidence for this is the excellent correlation between the mole fractions of cholate required to inactivate the receptor and to cause stripping of annular lipid. Under conditions where inactivation occurs, the concentration of detergent is likely to be sufficiently high as to favor formation of lipid–detergent and pure detergent micelles. The annular lipids could be extracted from the mixed lipid–receptor–detergent micelles, and the receptors become exposed to a micellar environment containing a high mole fraction of detergent (Volwerk et al., 1987).

Analysis of the amino acid sequence of the AChR shows that each subunit polypeptide contains at least four hydrophobic regions, each of which is postulated to span the membrane as an α helix [reviewed in Popot and Changeux (1984)]. Photoaffinity labeling studies suggest that helix 4 is at the lipid–protein interface (Giraudat et al., 1985). Removal of annular lipids might cause exposure of the hydrophobic receptor surface to the aqueous medium which would be energetically unfavorable. Since the present work deals with inactivation of detergent-solubilized receptors, exposure of hydrophobic surfaces on the receptor may arise either through an inability of the detergent to act as a protective seal against the aqueous environment or as a result of removal of detergent during dialysis. In either event, the protein must readjust itself within the membrane or micelle in order to lower the total free energy of the system. The readjustment process may be manifested either as changes in the secondary structure of the membrane-spanning portions of the receptor or through protein aggregation. Deformations in receptor secondary structure are anticipated to have a large effect on the functional properties of the ion channel since there is considerable evidence that the AChR ion channel spans the membrane (Lewis & Stevens, 1983; Popot & Changeux, 1984) and changes in AChR secondary structures in different lipids have been detected by FTIR spectroscopy (Fong & McNamee, 1987).

Irreversible loss of receptor activity might result also from unfavorable protein–protein contacts. Since the *Torpedo* AChR is primarily dimeric due to a covalent disulfide linkage between δ subunits, some protein–protein contact is inevitable. However, both the monomeric and dimeric forms of the receptor appear to be functionally equivalent (Anholt et al., 1980). Using (Ca^{2+} + Mg^{2+})-ATPase as a model protein, East et al. (1985) showed that significant protein–protein contact would only occur at lipid to protein ratios at, and below, the number of annular lipids (about 30 per ATPase). Consequently the functional effects of a variable aggregation state are most likely to be revealed in the types of delipidation experiments described here. Since the functional properties of both the ATPase and AChR are inhibited at lipid to protein ratios below the postulated number of annular sites, one possibility is that increases in random protein–protein contacts inactivate AChR.

Summary

The observation that a minimum number of lipids are required for function has several important consequences. For example, a great many membrane proteins have been shown to be irreversibly inactivated by detergents. It seems likely that such inactivation results from mechanisms similar to those described here. Our results suggest that it is not the absolute concentration of detergent but rather the detergent concentration corrected for the concentration of lipid in the system that is important. These observations have profound impli-

cations for reconstitution studies. By carefully controlling the membrane and the detergent concentrations, it may be possible to avoid irreversible inactivation of AChR by cholate. It may also be possible to use other detergents that could stabilize the AChR in a conformation that can be activated even after the lipids are removed. In principle, a similar approach can be applied to reconstitution of other membrane proteins using other detergents and lipids.

Registry No. DOPC, 10015-85-7; Affi-Gel 401, 78690-03-6; bromoacetylcholine bromide, 22004-27-9; Affi-Gel 401 carboxymethylcholine, 113597-80-1.

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