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Two pools of cholesterol in acetylcholine receptor-rich membranes from *Torpedo*

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The acetylcholine receptor (AChR)-containing electroplax membranes from *Torpedo californica* have a relatively high cholesterol content. Reconstitution studies suggest that this cholesterol may be important in preserving or modulating the function of the acetylcholine receptor-channel complex. We have manipulated cholesterol levels in intact *Torpedo* AChR-rich membrane fragments using small, unilamellar phosphatidylcholine liposomes. Conditions have been established that allow further subfractionation of sucrose gradient purified *Torpedo* electroplax membranes into AChR-rich and ATPase-rich populations and that, at the same time, achieve cholesterol depletion without phospholipid back exchange or fusion. The incubation of membranes with excess liposomes could only achieve about a 50% reduction in the molar ratio of cholesterol to phospholipid. In no case was the number of cholesterol molecules per AChR oligomer reduced below 36. The remaining cholesterol could not be depleted either by longer incubations or by multiple, sequential depletions. Cholesterol depletion was accompanied by a significant increase in bulk membrane fluidity as measured by electron spin resonance spectroscopy, but the equilibrium binding parameters of acetylcholine to its receptor were unaltered. This suggests strongly that there exist two pools of cholesterol in the AChR-rich *Torpedo* electroplax membrane: an easily depleted fraction that influences bulk fluidity, and a tightly-bound fraction perhaps surrounding the AChR oligomer.

Introduction

The local membrane environment experienced by the ACh receptor-channel complex may be crucial to its physiological function. Delipidation of purified ACh receptor-channel complex alters kinetic and allosteric properties of the receptor component [1–3] while relipidation restores these properties to an extent dependent both on the type and amount of phospholipid supplied [4]. Channel function is clearly dependent on membrane insertion, but may additionally be affected by varying the lipid composition of reconstituted membranes [4–9].

Receptor-rich electroplax membranes from *Torpedo californica* have, like many other plasma membranes, a relatively high cholesterol to total lipid content ($C/(C+P) = 0.25–0.52$ [10–12]), as do those from *Torpedo marmorata* (0.47 [13]), and the electric eel, *Electrophorus electricus* (0.63 [14]). The purified ACh receptor-channel complex preferentially incorporates into cholesterol-rich lipid

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monolayers [13], and the inclusion of cholesterol (or structural analogs) in lipid mixtures used for reconstitution facilitates AChR incorporation and subsequent vesicular sealing into these artificial membranes [7,8,15,16].

Data from reconstituted systems do not agree on the role of membrane cholesterol in regulating receptor and channel functions. (For a review, see Ref. 8.) While conductance has been observed in the absence of exogeneous cholesterol [17], other work with reconstituted planar bilayer systems [9,17,18] suggests an enhancement of the conductance and cooperativity of ligand-induced currents with an increase in membrane cholesterol. Kilian et al. [4], Dalziel et al. [5], Criado et al. [7] and Fong and McNamee [19] have all reported cholesterol-dependent changes in the ion gating function of reconstituted AChR-rich vesicles, despite conflicting conclusions regarding the nature [6,16] and magnitude of the cholesterol-mediated effect. In native vesicles, when endogenous cholesterol is diluted with added *Torpedo* phospholipid, the apparent affinity of the ligand for activating channels increases [20]. The role of cholesterol in slow desensitization, which can be monitored as a low to high affinity state transition, is also uncertain. Some studies suggest that the presence of cholesterol is unnecessary for the maintenance of the affinity state transition [5,19], whereas others emphasize the importance of cholesterol in efficiently maintaining this transition. Such lack of agreement may stem from problems inherent in the reconstitution system itself, and from the varying experimental techniques used [8].

Another approach to this problem is to manipulate cholesterol levels in intact AChR-rich membrane fragments using liposomes. Middlemas et al. [20] diluted cholesterol content relative to total lipid by fusing membranes with *Torpedo* phospholipid liposomes, leaving the cholesterol to AChR ratio unchanged. We have, instead, effected direct cholesterol removal by phosphatidylcholine liposome-mediated depletion of cholesterol [21]. Our results suggest strongly that the native AChR-channel complex is surrounded by tightly-held cholesterol which presumably buffers function of this integral oligomer from changes in bulk membrane fluidity.

Materials and Methods

Preparation of receptor-rich membranes. The procedure used was an adaptation of the method of Cohen et al. [22]. All procedures were carried out on ice and all solutions contained 0.02% sodium azide. Portions of skinned fresh electroplaque tissue dissected from chilled *Torpedo californica* (Pacific Biomarine, Venice, CA) were added to two volumes of distilled water and homogenized in a Virtis Hi-Speed 45 homogenizer (Virtis Company, Gardiner, NY) for 2 min at 95% maximum speed. After 30 s this process was repeated for 1 min. The homogenate was then centrifuged at $5000 \times g$ for 10 min and the supernatant set aside. The pellet was resuspended and centrifuged as above. The combined supernatants were filtered through gauze and centrifuged at $15\,000 \times g$ for 90 min. The pellets were resuspended as above. The yield from each 400 g of wet tissue was split into six 56 ml aliquots, each layered on 14 ml of 1.08 M sucrose and centrifuged at $80\,000 \times g$ for 90 min. Each pellet was resuspended in 23 ml of water and placed on a density gradient formed by freezing and thawing 1.2 M sucrose twice. After centrifugation at $80\,000 \times g$ for 4 h, 1 ml fractions were collected and assayed for acetylcholine binding sites and protein concentration [23]. The pooled receptor-containing fractions from 400 g of wet tissue typically yield 24 ml of suspension in 1.2 M sucrose containing 3–10 μM acetylcholine binding sites with a specific activity of 1–2 μmol of receptor per g protein. This suspension was stored under nitrogen at 4°C.

Binding of [^{3}H]acetylcholine. An aliquot of stock receptor suspension was diluted with an equal volume of water, pelleted at $80\,000 \times g$ for 70 min and resuspended in *Torpedo* Ringers solution (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM sodium phosphate, pH 7.0). A 30 min incubation with 10^{-4} M diisopropylfluorophosphate at room temperature proved sufficient to render esterase activity undetectable. [^{3}H]Acetylcholine solution was then added. Duplicate samples were withdrawn for counting on a Beckman LS 8100 scintillation counter after a further 30 min and the membranes were separated from the supernatant either by centrifugation at $100\,000 \times g$ for 90 min or by filtration

using 25 mm diameter GF/F glass fiber filters (Whatman, Clifton, NJ). The bound concentration was determined as the difference between total and free concentration and was corrected for non-specific binding. (For further details, see Ref. 23.)

Cholesterol loading of membranes. [^{3}H]-Cholesterol loading for purposes of establishing standard depletion conditions was achieved by incubating receptor-rich membranes for 2 h at room temperature with [^{3}H]cholesterol (added in ethanol) to a final concentration of one [^{3}H]cholesterol per 100 phospholipids in the membrane. [^{3}H]Cholesterol-loaded membrane was separated from free [^{3}H]cholesterol on sucrose step gradients (1.5 M, 0.9 M sucrose) centrifuged at 30000 $\times g$ for 90 min.

Preparation of liposomes. Egg phosphatidylcholine (EPC: Lipid Products, England) containing one mole percent of [^{14}C]phosphatidylcholine (algal PC, New England Nuclear, Boston) in chloroform was dried down under vacuum, resuspended in calcium-free *Torpedo* Ringers and sonicated intermittently at 4°C under nitrogen. (Heat Systems Model W185, using a microprobe. One minute on followed by one minute off for 60 min.) Finally the lipid suspension was clarified by centrifugation (15000 $\times g$, 20 min).

Incubation of receptor-rich membranes with liposomes. Receptor-rich membranes and varying excess of liposomes (on a lipid wt : wt basis) were incubated in Ca^{2+}-free *Torpedo* Ringers for varying lengths of time at 20°C in a water bath with periodic mixing (10-min intervals) by inversion of the tube. Optimal incubation conditions for cholesterol depletion were determined (see Results) to be 3 h with 10-fold excess of liposomes (wt : wt) and are henceforth referred to as 'standard conditions'.

Separation of incubated membranes from liposomes. Liposomes were separated from receptor-rich membranes following incubation by velocity sedimentation centrifugation in a swinging bucket rotor at 80000 $\times g$ for 4 h at 4°C on sucrose step gradients (2 ml each of 1.5, 1.4, 1.3, 1.2, 1.1, 1.0, and 0.8 M sucrose in Ca^{2+}-free *Torpedo* Ringers, in a 50 ml polypropylene centrifuge tube). Liposome-membrane suspensions were carefully layered above the last 0.8 M sucrose step, covered with Ca^{2+}-free *Torpedo* Ringers, and collected in fractions.

Spin-labeling studies. Torpedo AChR-rich membranes were spin-labeled for electron spin resonance (ESR) spectroscopy by gently shaking with 5-(2,2-dimethyl-*N*-oxyloxazolidine)-palmitate (5-doxylpalmitate). Aliquots of 5-doxylpalmitate were deposited as a thin film from methanolic solution, and vacuum dried. Membranes were added to a final concentration of spin label of 1 mole percent in membrane lipid. After incubation (6 h, 4°C) samples were transferred to thin walled glass tubes (1–3 mm i.d.).

Spectroscopy was performed on a Varian E-109 ESR spectrometer operating at 9.2 GHz, microwave power of 10 mW, magnetic field strength of 3250 Gauss and modulation amplitude of 1.0 Gauss. The temperature in the ESR sample cavity was monitored by a thermistor and maintained at 20.0 ± 0.1°C by a stream of thermostated N_2 passing through a Dewar insert containing the sample. Spectra were obtained (filter time constant of 1 s, scan time of 8 min) and splittings were measured directly from the graduated chartpaper. The lipid order parameter (S) was calculated by the method of Hubbell and McConnell [24] with spectral width corrections for solvent polarity [25]. Four samples from each type of membrane were analyzed, and the mean lipid order parameters calculated.

Assays. Cholesterol was determined either enzymatically by the method of Allain et al. [26], using reagents purchased from Supelco Inc. (Bellefonte, PA) or chemically by the method of Ruddell and Morris [27]. Protein was determined by the method of Hartree [28]. Phospholipid was determined as organic phosphate by the method of McClare [29]. The mean molecular weight of the phospholipids was assumed to be 790 daltons. In experiments in which phospholipid was determined, Tris-HCl was substituted for sodium phosphate in *Torpedo* Ringers. Acetylcholinesterase was assayed by the method of Ellman et al. [30]. Quantitative protein characterization of the various membrane fractions was by SDS-polyacrylamide gel electrophoresis and subsequent Coomassie blue staining and densitometry [31].

Results

Incubation conditions for cholesterol depletion

The rate of egg PC liposome-mediated cholesterol depletion should be limited by desorption of cholesterol from the biomembrane into solution in the aqueous phase, provided that the acceptor vesicle concentration is high enough for rapid absorption of cholesterol monomers from the aqueous phase [32,33]. Furthermore, liposomes may associate with membranes over time [20,34]. The kinetics of cholesterol-liposome exchange at room temperature were, therefore, followed to select optimal incubation times allowing efficient exchange with minimum liposome-membrane association. Under conditions where receptor membranes were in 14-fold excess over liposomes, cholesterol equilibrated between the two membranes within about 2 h at 20°C. Furthermore, when liposomes were in 10-fold excess, little advantage was found in continuing the incubation beyond 2–3 h. On this basis, we chose 3 h at 20°C as the standard incubation condition.

A series of experiments with increasing liposome concentration were performed in order to maximize cholesterol depletion while minimizing other effects, such a back exchange of liposome phospholipid into receptor membranes. In this series, receptor membranes were pre-labeled with [^{3}H]cholesterol and the liposomes prepared with [^{14}C]phosphatidylcholine. Following standard incubation, no significant amounts of [^{14}C]phospholipid were detected on a density gradient at 1.2 M sucrose, the density at which receptor membranes band. This conclusion held at 10-, 50-, 75- and 100-fold excess of liposomes over receptor (10 μg protein/ml), and since the [^{3}H]cholesterol extraction efficiency did not vary over this range, a 10-fold excess of liposomes was deemed a sufficient acceptor concentration in further experiments.

Liposome incubation induces receptor membrane subfractionation

A further reason for limiting acceptor concentration to a 10-fold excess was that after incubation [^{14}C]phosphatidylcholine, together with [^{3}H]cholesterol, appeared in a band below the sucrose-buffer interface but distinct from the major band of [^{14}C]phosphatidylcholine in the liposomes above the interface. The minor band was a small fraction of the total [^{14}C]phosphatidylcholine, but increased dramatically as the liposome excess was varied from 10- to 100-fold.

This phenomenon was further examined at higher membrane concentration (3–4 mg protein/ml) using three hour incubation with 10-fold excess of liposomes. Fig. 1 shows the results of one such incubation after sucrose density fractionation. The gradient separated the liposome-incubated receptor membranes into discrete light and heavy bands each containing both protein and acetylcholine receptors. The light band appeared as a thin sheet between 1.0 to 1.1 M sucrose, corresponding to the position at which the minor band was seen in the double label experiments described above. The [^{14}C]phosphatidylcholine marker included in the liposomes confirmed this assignment. Eight percent of this radioactivity was associated with this band, the remainder floating with the liposomes above the sucrose-buffer interface. This suggests that the density of this membrane population is reduced by some uncharacterized but intimate association with liposomes. Middlemas et al. [20] report a similar decrease in density following AChR membrane-phospholipid liposome fusion. This association was time-dependent, because when liposomes and membranes were mixed and then immediately centrifuged, only the heavy band at 1.2 M sucrose was observed. Incubations between zero and two hours produced steadily increasing amounts of light band at the expense of the heavy band, but between two and four hours the ratio of these two bands was unchanged. Although the nature of the liposome-biomembrane association is of interest in its own right, it was not the focus of this study and was not further investigated.

While the heavy band was depleted of cholesterol relative to the control membranes, the possibility arises that rather than depleting cholesterol from receptor membranes, liposome incubation simply effected a separation of two pre-existing native membrane subpopulations of differing cholesterol content. Or indeed, both processes could be in operation simultaneously. Preliminary analysis showed that 80–85% of measurable ATPase activity resided in the light band. This

observation was confirmed by the distribution of the 90 kDa polypeptide following SDS-polyacrylamide gel electrophoresis of the two membrane fractions.

The above ATPase distribution was reminiscent of that obtained by Jeng et al. [35] from a similar starting population of native AChR-rich membranes which they subfractionated by a velocity sedimentation procedure. Accordingly, we fractionated our native membranes into the two subfractions of Jeng et al. [35] and performed the following cross-over experiment. Both Jeng's [35] upper receptor-rich fraction and their lower ATPase rich band and an equal mixture of the two bands were incubated for three hours with 10-fold excess of liposomes and then fractionated on sucrose gradients together with controls that had not been exposed to liposomes. The upper receptor-rich fraction banded at 1.2 M sucrose (compare heavy fraction; Fig. 1) together with a faint band at the 1.0–1.1 M sucrose interface (see Fig. 2A) where our light fraction appeared in Fig. 1. For comparison, when this receptor-rich fraction was centrifuged without prior incubation with liposomes, a single band appeared at 1.2 M sucrose and no material was observed at lower densities.

The behavior of the lower, ATPase-rich, band contrasted dramatically (Fig. 2B). Whereas the control membranes also banded at 1.2 M sucrose, the liposome-incubated sample sedimented almost completely within the 1.0 M sucrose fractions and assumed the characteristic thin sheet-like appearance of our original light band. Finally, the pooled fractions separated into the expected heavy and light fractions following incubation with liposomes (Fig. 2C).

Thus, velocity sedimentation fractionates native membranes into the same two subpopulations revealed by liposome incubation, and the receptor-rich fraction of Jeng et al. [35] provides us with

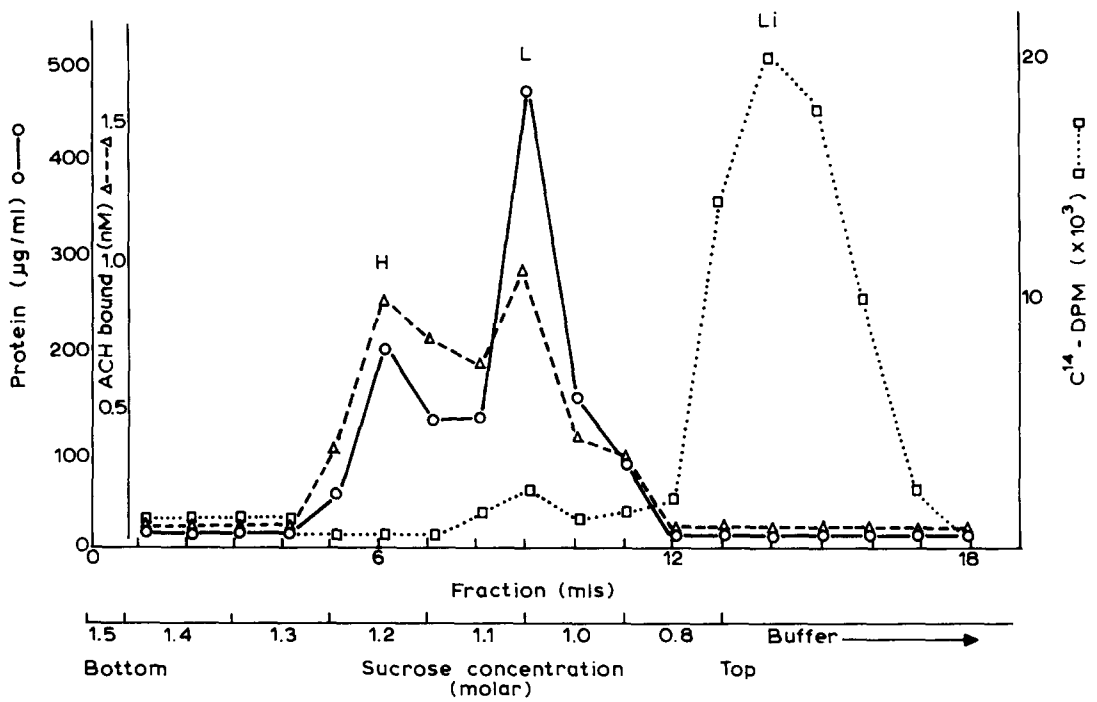
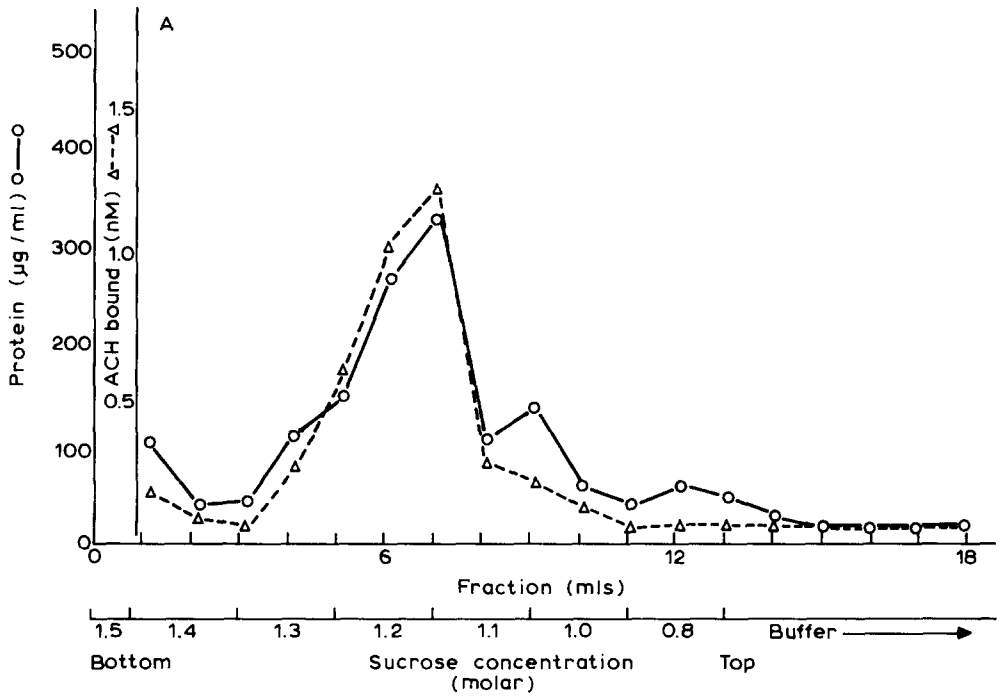


Fig. 1. Sucrose density fractionation of liposome-incubated AChR-rich membranes. AChR-rich membranes were incubated for 3 h at 20°C with 10-fold excess [^{14}C]phospholipid labeled egg PC liposomes and then separated from liposomes by velocity sedimentation at 80000× *g* for 4 h at 4°C on 1.5–0.8 M sucrose step gradients as detailed in Methods. The ^{14}C-labeled liposomes primarily float at or above the 0.8 M sucrose -Ca^{2+}-free *Torpedo* Ringers interface (Li; fractions 12–18), whereas the membranes subfractionate into heavy (H; 1.2 M sucrose; fractions 5–7) and light (L; 1.0–1.1 M sucrose; fractions 8–12; 8% of total radioactivity) components.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Protein (µg/ml) o—o
ACH bound (nM) Δ---Δ
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400
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200
100
0
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Fraction (mls)
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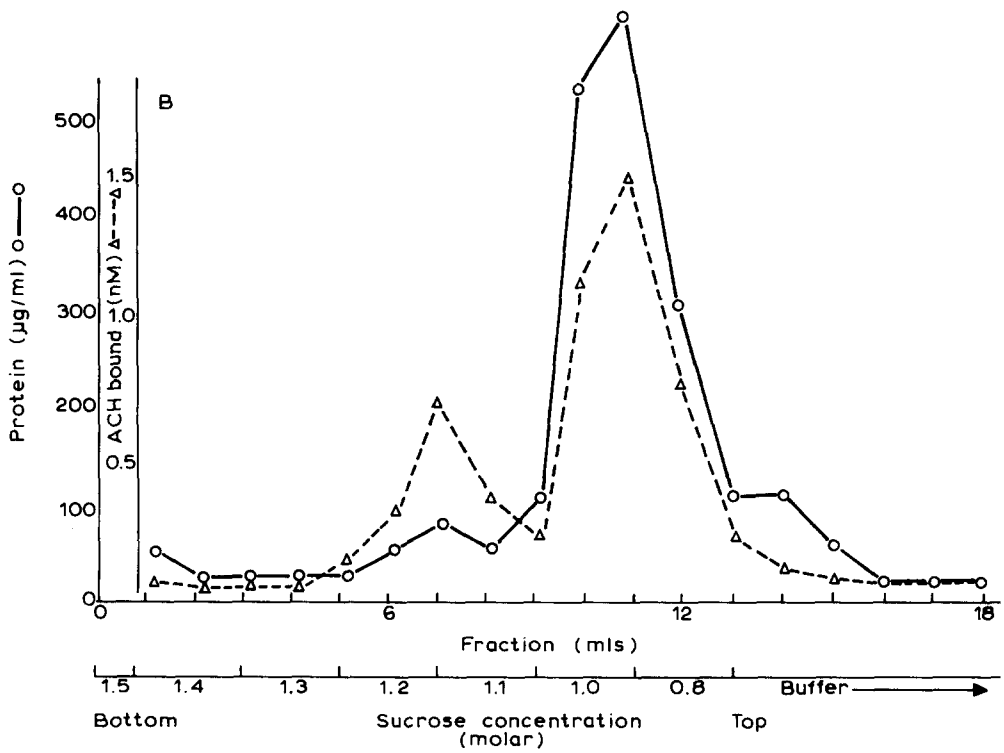
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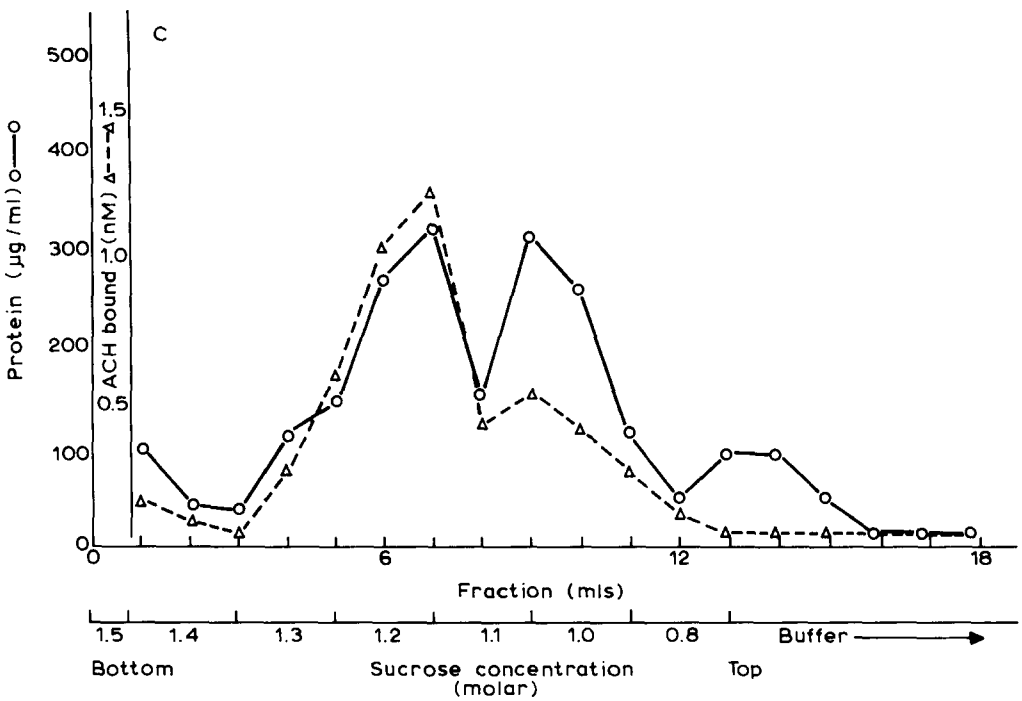

Fig. 2. Cross-over experiment utilizing Jeng et al.'s [35] membrane subfractions. Native membranes were subfractionated into 'upper' (receptor-rich) and 'lower' (ATPase-rich) fractions by the method of Jeng et al. [35] and then each fraction incubated separately with a 10-fold excess of liposomes for 3 h at 20°C. Finally, incubated membranes were separated from liposomes by velocity sedimentation (1.5–0.8 M sucrose in 2 ml steps, spun at 80000× *g* for 4 h at 4°C.). (A) Liposome-incubated AChR-rich membranes (Jeng's 'upper' fraction) band primarily at the 'heavy' position (1.2 M sucrose; see Fig. 1) with only slight contamination at the 1.0–1.1 M sucrose interface ('light'). (B) Liposome-incubated ATPase-rich membranes (Jeng's 'lower' fraction) band primarily at the 'light' position (see Fig. 1) with only slight contamination by 'heavy', AChR-rich membranes (fractions 6–8). (C) Liposome-incubated pooled Jeng's 'upper' and 'lower' membranes sort into 'heavy' and 'light' fractions as expected.

the control composition of the heavy fraction of membranes before liposome incubation. The composition of velocity fractionated membranes is given in Table I. The receptor specific activity of the upper phase is over three times that of the lower phase, whereas the lower phase has about 3.5-fold higher ATPase activity than the upper. The lower phase has more lipid molecules per acetylcholine receptor, but the cholesterol to total lipid ratios are similar in all fractions and espe-

TABLE I

COMPOSITION OF MEMBRANES PURIFIED BY VELOCITY SEDIMENTATION ACCORDING TO REF. 35

	AChR [a]/protein (nmol/mg)	Mole ratios		Percent cholesterol to total lipid [b]	ATPase % of total	AChE (mmol/h per mg) (×10^3)	Order parameter [c]
		Chol/AChR	PL/AChR				
Sample applied	1.52	237	399	37	100.0	11	0.683 ± 0.002
Upper phase (receptor-rich)	1.74	120	223	35	20.4	4.9	0.682 ± 0.003
Lower band (ATPase-rich)	0.52	709	994	42	69.1	4.9	0.670 ± 0.003

[a] AChR are [^{3}H]acetylcholine binding sites.
[b] 100 ($C/(C + P)$).
[c] 5-Doxylpalmitate at 20°C.

cially so in the unfractionated and receptor rich fractions.

Cholesterol depletion

On a molar basis cholesterol was about one third of the total lipids in the control membranes (Tables I and II), in good agreement with previous workers [10–12]. Velocity fractionation of receptor-rich membranes shows the upper fraction to have essentially the same ratio (Table I). Thus, either membrane may be used as a control for estimating the degree of cholesterol depletion relative to total lipid. When native membranes are cholesterol depleted and then fractionated by centrifugation the results shown in Table II are obtained. As expected, the heavy band showed no change in the molar ratio of phospholipid to receptor when compared to undepleted velocity fractionated receptor-rich membranes (Table I). The ratio of cholesterol to total lipid has, however, fallen from 33% to 20%. The depleted membranes in Table II have 51 moles of cholesterol per mole of [^{3}H]acetylcholine sites. In nine depletions the final mean cholesterol content was 48 ($\pm$26) per [^{3}H]acetylcholine site ($\pm$S.D.). The experiment in Table III shows the highest cholesterol content and the lowest content was 18.

In Table II, roughly 40% of the cholesterol was removed by incubation with a 10-fold excess of egg liposomes for 3 h; clearly a statistical re-distribution of cholesterol between the biomembranes and the liposomes has not been achieved. To further investigate this point, membranes were serially incubated with liposomes and fractionated by centrifugation between each incubation. Standard incubation conditions were employed and samples were removed for analysis after all but the second fractionation and the remaining sample stored overnight under nitrogen at 4°C. Starting from native membranes, a total of four depletions were performed on successive days. The results are shown in Table III. The cholesterol concentration per unit weight of membrane protein fell after the first depletion, but had increased after the third depletion. This paradoxical increase resulted from a decrease in membrane protein in successive heavy bands as evidenced by a decrease in ATPase and acetylcholinesterase activities and an increase in acetylcholine receptor specific activity. The loss of ATPase suggests that membrane fractionation is occurring, but we cannot rule out removal of extrinsic membrane proteins by liposomes because the acetylcholinesterase is not depleted in parallel with the ATPase. On the basis of the mole ratio of cholesterol per acetylcholine receptor, 94% of the cholesterol depletion occurs in the first depletion.

Cholinergic ligand binding to cholesterol depleted membranes

To examine whether cholesterol depletion had significantly altered the functional properties of the receptor, [^{3}H]acetylcholine binding was examined over a concentration range from 2.5 to 150 nM. Non-specific binding was unaltered by any of the treatments. Analysis of the dependence of displaceable [^{3}H]acetylcholine binding on the free [^{3}H]acetylcholine binding yielded curves that could be fitted to a Hill equation. Control membranes had a Hill coefficient of 1.46 $\pm$ 0.076 (S.D.) and a half binding concentration of 12.1 $\pm$ 0.71 nM, values which are within the range of those

TABLE II

CHANGE IN CHOLESTEROL UPON INCUBATION WITH LIPOSOMES

	AChR [a]/ protein (nmol/mg)	Mole ratios		Percent cholesterol to total lipid [b]	Order parameter [c]
		Chol/ AChR	PL/ AChR		
Control	1.32	206	424	33	0.683 $\pm$ 0.002
Depleted	1.27	51	204	20	0.651 $\pm$ 0.002

[a] AChR are [^{3}H]acetylcholine binding sites.

[b] 100 ($C/(C + P)$).

[c] 5-Doxylpalmitate at 20°C.

TABLE III

CHANGE IN MEMBRANE COMPOSITION FOLLOWING REPEATED INCUBATION WITH 10-FOLD EXCESS OF PHOSPHATIDYLCHOLINE LIPOSOMES FOR 3 h AT 20°C

Membrane source	AChR [a]/ protein (nmol/mg)	Chol/ protein (μg/mg)	Chol/ AChR (mol/mol)	ATPase (A/min per mg)	AChE (mmol/h per mg) ($\times 10^3$)
Control	1.44	88	157	0.5421	16.8
Depleted 1×	2.36	73	80	0.1059	11.6
Depleted 3×	3.18	106	86	n.d. [b]	5.4
Depleted 4×	4.50	125	73	n.d. [b]	5.4

[a] AChR are [^{3}H]acetylcholine binding sites.
[b] n.d., not detectable.

reported in the literature [23,36]. Membranes depleted of cholesterol and collected in the 1.2 M sucrose fraction had essentially identical binding parameters of 1.49 ± 0.050 and 11.1 ± 0.050 nM, respectively.

Lipid order in cholesterol depleted membranes

The order parameter reported by 5-doxyl-palmitate at 20°C was 0.683 in the control membranes which banded at 1.2 M sucrose. When these membranes were fractionated by the method of Jeng et al. [35] the receptor-rich fraction had the same order parameter as the controls (Table I), but when fractionation was carried out after incubation with liposomes, the cholesterol depleted, heavy fraction's order parameter was decreased 0.032, or 4.7% (Fig. 3). The light fraction was not examined because of its heterogeneous nature. However, the ATPase-rich fraction of Jeng et al. [35] had a slightly lower order parameter than the parent membrane (Table I).

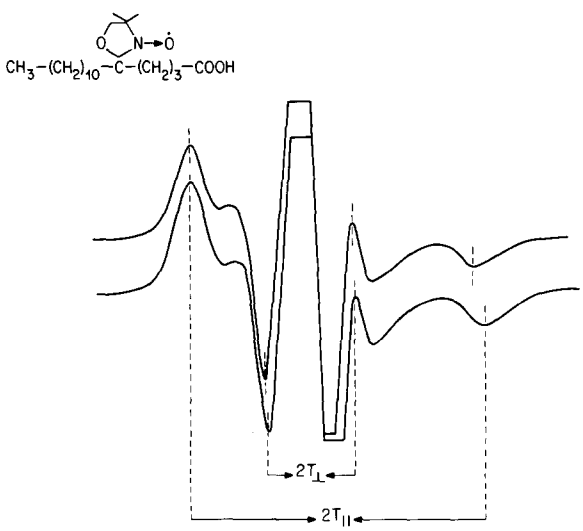

Fig. 3. ESR spectra of normal and cholesterol-depleted AChR-rich membranes from the electroplaques of *Torpedo*. Representative spectra were obtained at 20.0 ± 0.1°C from cholesterol-depleted membranes (top) and controls (bottom) (Table II), spin-labeled with 1 mole percent 5-doxylpalmitate. The low-field peaks are aligned to facilitate comparison. (Magnetic field strength: 3250 G; modulation amplitude: 1.0 G; modulation frequency: 100 kHz. Microwave power: 10 mW; Frequency: 9.2 GHz. Filter time constant: 1 s; scan range: 100 G; scan time: 8 min.)

Discussion

We have established conditions for the liposome-mediated manipulation of cholesterol in native receptor-rich electroplax membranes without back exchange of phosphatidylcholine. That fraction of cholesterol available for exchange is almost completely depleted within a 3 h incubation period with a 10-fold excess (wt : wt) of liposomes. Phosphatidylcholine liposomes have been successfully employed to add or deplete plasma membrane cholesterol in a number of other experimental systems [20,37–40]. A limit to the amount of membrane cholesterol that can be removed with this approach has usually been reported. In erythrocytes, for example, Giraud and Claret [37] report a 40% maximal depletion of cholesterol (relative to total lipid), while Poznansky and Czekanski [39] claim substantially more (80%), yet both agree that a significant fraction of the plasma membrane cholesterol pool is unavailable for depletion.

Failure to completely deplete cholesterol could

result either from compartmentalization preventing access of all the cholesterol to liposomes or from a specific interaction between the unextracted cholesterol and another membrane component. However, slow diffusion of cholesterol across the bilayer (flip-flop) or limited liposome access are not likely explanations because measurements in other eukaryotic membranes often show rapid cholesterol flip-flop [41]. In the squid-axon, for example, flip-flop occurs with a half-time on the order of minutes ($t_{1/2} = 7.3–15.3$ min [38]). Since the serial liposome exposures reported in Table III were carried out at intervals of about 16 h, flip-flop would have to be almost non-existent to account for our data.

Alternatively, it has been suggested that alterations in the packing of cholesterol and phospholipid molecules below a critical $C/(C + P)$ ratio of 30 mole percent keeps cholesterol more tightly bound to membrane phospholipid and thus unavailable for exchange to phosphatidylcholine liposomes [37,39]. However, phosphatidylcholine and phosphatidylethanolamine account for over 80% of *T. californica*'s phospholipid and are unlikely to retain cholesterol significantly in the face of repeated exposure to a 10-fold excess of egg phosphatidylcholine. A third explanation is specific cholesterol-AChR oligomer interactions This is suggested by the observation that AChR prefentially incorporates into cholesterol monolayers [13] and by reconstitution studies reviewed in the Introduction.

In support of this hypothetical tight cholesterol–AChR interaction, cholesterol depletion caused no change in the equilibrium binding of acetylcholine to the desensitized receptor, despite a relatively large change of order parameter. However, alcohols and volatile anesthetics perturb equilibrium binding at comparable changes in order parameter [25]. One explanation would be that anesthetics perturb the boundary and bulk lipids whereas cholesterol depletion only changes order in bulk lipid.

In contrast to the slow conformational change associated with desensitization, the more rapid changes associated with channel opening appear more sensitive to cholesterol manipulation. Thus, decreasing the cholesterol to phospholipid ratio by fusing AChR-rich membranes with *Torpedo* phospholipid liposomes causes an increase in carbachol's apparent affinity for stimulating ion flux [20]. That gating is more sensitive than slow agonist induced desensitization to lipid environment has been noted in some reconstitution studies [7], but the explanation remains obscure. On the other hand, manipulation of the cholesterol to phospholipid ratio in *Xenopus* myocytes did not change channel conductance and revealed little or no dependence of the single channel burst duration on the cholesterol level [40].

Opposed to the concept of cholesterol–acetylcholine receptor interactions are studies using the antibiotic filipin, which interacts specifically with cholesterol and can be visualized as 20–40 nm pits in cholesterol-rich membranes. It fails to reveal cholesterol within postsynaptic receptor clusters of *T. marmorata* electroplax membranes [42], *Xenopus* neuromuscular junctions [43] or chick myotubes [43]. In contrast, filipin- and saponin-cholesterol complexes have been demonstrated both in the surrounding bulk membrane and to a lesser extent within AChR clusters in rat myotubes [44].

However, the tight packing of receptor proteins (9 nm center-to-center distance) may sterically interfere with filipin-cholesterol pit formation and detection. In support of this saponin mediated an increase in the permeability of receptor-rich microsacs (*T. californica*) to lactoperoxidase [45], and ring-like substructures induced by saponin were detected in areas densely packed with acetylcholine receptor rosettes [46]. Morever, cholesterol diazoacetate, a photoaffinity probe, labeled all four AChR subunits in AChR-rich membranes from *Torpedo* [20]. Indeed, it has been suggested recently that a negative response to filipin is not sufficient to demonstrate low membrane sterol concentrations, particularly in membrane domains characterized by closely-associated proteins [46]. Thus, the contrary studies with antibiotics need not be given much weight.

More direct support for the preferential inclusion of cholesterol in the boundary region comes principally from spin-label studies. The presence of a spin-labeled cholesterol analog (androstane) in the boundary of native membranes was clearly demonstrated [48]. The molar proportion of the total label in the boundary was about half. Since

the specific activity of the receptor in the membranes was similar to ours, the label was clearly not randomly distributed between fluid and immobilized components. Indeed, in reconstituted systems, androstane has a partition coefficient of about four in favor of the boundary, whereas for phospholipid spin labels this ratio is close to one [49].

Is the number of cholesterol molecules retained in our cholesterol-depleted membranes consistent with the hypothesis that they are mainly located in the boundary layer of the receptor? Two independent structural studies [50,51] show the receptor is a cylinder with a diameter of 60–65 Å in the plane of the bilayer. The surface area of cholesterol (38–39 $Å^2$) and phospholipids (60–80 $Å^2$, depending on the head group and degree of unsaturation) is known from monolayer studies [52,53]. Assuming simple cylindrical geometry then 44–53 phospholipids or 60–65 cholesterol molecules can be packed in two complete monolayers around the acetylcholine receptor. The exact lipid-protein interface can only be crudely estimated by these calculations which assume a smooth surface and ignore interstitial lipid, dimerization of the oligomeric units, protein-protein aggregation and the possible interaction of the surface protrusion, with a diameter of some 85 Å, with lipid head groups [50]. Nonetheless, these estimates are close to those obtained by electron spin resonance studies in reconstituted membranes [49].

Receptor-rich membranes contain about 450 phospholipid and 240 cholesterol molecules per oligomer (Table I. Each receptor oligomer contains two binding sites for acetylcholine), so the majority of these lipid molecules will not be adjacent to the receptor. On the other hand, after depletion, the number of cholesterol molecules per acetylcholine receptor oligomer in our membranes ranged from 36 to 172. In three of nine depletions there was considerably less cholesterol than that required for about one bilayer of nearest neighbors. If we take the most complete depletion as indicating an upper limit for the number of cholesterol 'sites' per receptor oligomer we can calculate approximately the equilibrium constant, K, for cholesterol between the bulk bilayer and the 'boundary layer' [54]. Geometric arguments similar to those above indicate that there would be room for 16 phospholipids in addition to the 36 cholesterols next to each receptor. Assuming this composition in the boundary layer, the data for the receptor rich membranes in Table I and the depleted membranes in Table II yield an estimate of between 5 and 15 for the equilibrium constant, K. These figures are upper limits. Considering the assumptions made and the degree of purity of the membrane protein this estimate can be considered in satisfactory agreement with the value of $K = 4.3$ determined spectroscopically for androstane in reconstituted receptor membranes [49].

What could the structural role of cholesterol be at the lipid/protein interface? In many proteins cholesterol is excluded from this interface and the acyl chains of the phospholipids take up disordered arrangements on the protein surface [55]. This disordering probably occurs as the flexible fatty acid chains attempt to maximize short range lipid-protein intermolecular forces on the 'rough' surface of the protein. On the other hand, the relatively inflexible cholesterol molecule opposed to a 'rough' protein surface would create energetically unfavorable voids. Thus, in those membrane proteins where cholesterol is favored at the lipid/protein interface, one might expect to find specific features, perhaps sites or clefts, of well-defined and complementary geometry. It seems unlikely that these would be associated with relatively rigid alpha-helices. Interestingly, Williams [56] has suggested that steroids, like hemes and flavins can provide hydrophobic platforms around which proteins can fold to stabilize a particular conformation. At the current level of resolution structural studies [50,51] leave the nature of the transmembrane region of the acetylcholine receptor in considerable doubt [57], but the requirement of cholesterol for pore formation by amphotericin B provides a useful, if limited analogy [58].

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