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RECONSTITUTION OF ACETYLCHOLINE RECEPTOR FUNCTION IN LIPID VESICLES OF DEFINED COMPOSITION

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The effect of specific lipids on the functional properties of the acetylcholine receptor were examined in reconstituted membranes prepared from purified *Torpedo californica* acetylcholine receptor and various defined lipids. Cholesterol and negatively charged lipids greatly enhanced the ion influx response of the vesicles as measured by the effect of a receptor agonist on cation translocation across the vesicles. Part of the lipid-dependent effects could be attributed to alterations in the average size of the vesicles. All lipid mixtures used permitted complete incorporation of receptor and retention of ligand binding properties. Quantitative differences in ion flux properties suggest a modulating role for specific lipids in acetylcholine receptor function.

Introduction

The nicotinic acetylcholine receptor is a transmembrane protein found at vertebrate neuromuscular junctions and at synapses in the electric organs of certain electric fish, such as the electrical eel (*Electrophorus electricus*) and various marine rays (*Torpedo* species). The acetylcholine receptor specifically binds cholinergic activators and transduces the binding into a large increase in cation permeability through a receptor-associated ion channel. In the prolonged presence of activators, the receptor channel becomes inactivated, a reversible phenomenon known as desensitization. All of these functional properties are preserved in membrane vesicles isolated from electric organs, and sensitive assays have been developed to measure ligand binding, flux activation and desensitization (for reviews, see Refs. 1–3). *Torpedo*-derived membranes have emerged as a preferred choice for detailed biochemical and biophysical studies, since membranes containing up to 50% of their total protein as functional acetylcholine receptor molecules can be isolated [3].

Using *Torpedo* membrane vesicles, Andreasen and McNamee [4] showed that the ion permeability control properties of the receptor were exquisitely sensitive to lipid perturbations brought about by treatment with phospholipase A_2. Complete inhibition of agonist-stimulated ion flux was observed at low phospholipase A_2 concentrations that caused only partial hydrolysis of phosphatidylethanolamine and no hydrolysis of phosphatidylcholine. Andreasen and McNamee suggested that the lipid protein interface might provide a preferred site for enzyme action. In further studies, they showed that unsaturated fatty acids

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Abbreviations: carbachol, carbamylcholine chloride; DOPC, dioleoylphosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PMSF, phenylmethylsulfonyl fluoride; Mops, 4-morpholinepropanesulfonic acid.

(one of the phospholipase hydrolysis products) could also reversibly block ion flux responses [5,6]. Interestingly, the fatty acids had no effect on the ligand-binding properties of the receptor, including a ligand-induced binding affinity transition that is generally assumed to be associated with desensitization [7]. Thus, the fatty acids appeared to block flux by a mechanism independent of the desensitization phenomenon.

Using biophysical techniques, Marsh et al. [8] and McNamee et al. [9] provided evidence that the lipids of the native *Torpedo* membranes existed in two distinct domains, as indicated by the electron paramagnetic resonance spectra of membrane-associated spin-labelled fatty acids and phospholipids. One of the spectral components was characteristic of a motionally restricted environment and suggested the presence of a 'boundary layer' [10] about the protein [8,9].

Although the *Torpedo* membranes represent one of the best systems for studying both membrane structure and function, they do have limitations. The complex lipid composition of the vesicles [11] limits the analysis of specific lipid effects, and the heterogeneity of the vesicle populations with respect to both size and protein components further complicates detailed analysis. A more subtle limitation arises from the high density of acetylcholine receptor sites on the surface of the membranes. Rapid kinetics measurements on the millisecond time scale can still only partially resolve the initial rates of ion flux, since the vesicles rapidly equilibrate with external ions in the presence of activators [12–14].

All of these limitations could be avoided by using a system in which both the protein and lipid composition were completely controlled. A major advance in this direction was achieved in 1978 when Epstein and Racker [15] first convincingly demonstrated that detergent-solubilized acetylcholine receptor could be reincorporated into lipid vesicles with recovery of ion permeability control properties. The lipid used was Asolectin, a crude soybean lipid preparation. The success of the approach of Racker can be appreciated by the speed with which many other groups reproduced and extended the observations [16–20]. Purified acetylcholine receptor can now be reconstituted into lipid vesicles and into planar bilayer membranes (for reviews, see Refs. 21, 22). Interestingly, the reconstitution requires that the receptor be maintained in the presence of lipids throughout all purification procedures, thus reemphasizing the importance of lipids in acetylcholine receptor function.

In order to extend the earlier biophysical and biochemical studies, we have begun to examine systematically the lipid requirements for successful reconstitution [18]. In this paper we analyze the effects of negatively charged lipids and cholesterol on the size and ion flux properties of reconstituted membrane vesicles containing purified acetylcholine receptor.

Materials and Methods

Acetylcholine receptor membrane preparation

Live *Torpedo californica* rays were obtained from Pacific Biomarine (Venice, CA) and killed immediately upon arrival, and the excised electroplax tissue was frozen in liquid nitrogen. A crude membrane fraction was prepared from the frozen tissue. Typically, 600 g tissue were thawed and homogenized with 600 ml homogenization buffer (10 mM sodium phosphate/5 mM EDTA/5 mM EGTA/10 mM iodoacetamide/0.1 mM PMSF/0.02% NaN_3, pH 7.5) with a Polytron PCU-2-110 in four 30 s bursts at setting 7. The homogenate was centrifuged for 10 min at 5000 rpm in a Sorvall GSA rotor and the supernatant was filtered through four layers of cheesecloth and saved. The pellet was rehomogenized in 200 ml homogenization buffer and centrifuged as above. All steps were carried out at 0–4°C. The pooled supernatants were centrifuged at 34000 rpm in a Type 35 rotor in a Beckman L5-65 centrifuge for 45 min. In experiments where the receptor was purified prior to reconstitution, the pellet was resuspended in approx. 75 ml buffer A (100 mM NaCl/10 mM Tris-HCl/0.1 mM EDTA/0.02% NaN_3, pH 7.4) and used as the starting material for cholate extraction (see below). For experiments in which acetylcholine receptor was extracted directly from membranes and used without additional purification, a more highly purified membrane fraction was prepared. Briefly, the high-speed pellet corresponding to 200 g original tissue was resuspended in 32 ml buffer comprising 28% w/w sucrose/10 mM sodium phosphate/0.1 mM

EDTA/0.02% NaN_3, pH 7.0. 8-ml aliquots were layered on top of a discontinuous sucrose gradient consisting of 10 ml 30% sucrose (w/w), 12 ml 35% sucrose (w/w) and 7 ml 41% sucrose (w/w), all in 10 mM $NaPO_4$/0.1 mM EDTA/0.02% NaN_3, pH 7.0. The samples were centrifuged at 26000 rpm in an SW 27 rotor for 4 h. The membrane band on top of the 35% sucrose layer was removed and diluted 1 : 1 with water and centrifuged at 35000 rpm in a Type 35 rotor for 35 min. The membranes were base-extracted by resuspending the pellet to approx. 0.5 mg/ml protein in water and adjusting the pH carefully to 11.0 with NaOH. The suspension was stirred for 30–45 min and then centrifuged at 35000 rpm for 45 min. The pellet was resuspended in buffer A.

Cholate extraction

Acetylcholine receptor-containing membranes (either the crude or highly purified fractions) corresponding to an original membrane preparation from 200 g tissue were diluted to 2 mg protein/ml in buffer A and 10% sodium cholate (w/v) in buffer A was added to give a final cholate concentration of 1%. The mixture was stirred for 30 min and then centrifuged at 35000 rpm in a Type 35 rotor for 30 min. The supernatant from highly purified membranes was used directly for reconstitution (see Reconstitution). The supernatant from crude membranes was applied at a flow rate of 1 ml/min to a 15 ml affinity column prepared from Affi-Gel 401 (Bio-Rad, Richmond, CA) and bromoacetylcholine [24]. The column was previously equilibrated with buffer A supplemented with 1% sodium cholate and 2 mg/ml dioleoylphosphatidylcholine (DOPC; Avanti Biochemicals, Birmingham, AL). The column was washed with 6–10 column volumes of buffer A containing the 1% cholate and 2 mg/ml DOPC and then eluted with 50 ml 10 mM carbamylcholine chloride (carbachol) buffer. The acetylcholine receptor emerged as a sharp peak just after the void volume of the column. Protein concentration during washing and elution was monitored by measuring absorbance at 280 nm. (Empirically, an absorbance of 1.0 corresponded to a value of 0.6 mg protein/ml as measured by the procedure of Lowry et al. [25].) The column could be used at least five times without noticeable loss in yield or quantity of acetylcholine receptor if it was washed extensively with buffer B and then with water containing 0.02% NaN_3.

The purified acetylcholine receptor (typically 1 mg/ml in approx. 10 ml buffer) was either used immediately for reconstitution or frozen in liquid N_2 after adding solid sucrose to give a 0.5 M solution.

Reconstitution of acetylcholine receptor in liposomes

The same general procedure was used for both membrane-extracted and purified acetylcholine receptor. Typically, 0.7 ml of the acetylcholine receptor cholate solution at a protein concentration of 1–1.65 mg/ml was mixed with 0.3 ml of a 65 mM lipid suspension (see below) in 4.16% cholate in buffer A. The entire mixture (2% final cholate concentration) was dialyzed for 48 h against 1000 vol. buffer A with three or four changes of buffer. The buffer was bubbled with N_2 for 10–15 min before use. In some experiments [^{14}C]cholesterol or [^{14}C]phosphatidylcholine was added to the lipids before dialysis and there was no detectable loss of lipids through the dialysis tubing.

Different procedures were used to prepare the lipid suspensions depending upon the lipids used. For Asolectin (Associated Concentrates, Woodside, NY) the desired amount was weighed, suspended in 1 ml 4.16% cholate and sonicated for 20 min under nitrogen. For purified phospholipids and cholesterol, the desired amounts were premixed as chloroform solutions, evaporated to dryness under nitrogen and then placed under vacuum at room temperature for at least 2 h to remove residual solvent. The dried lipids were then resuspended in 4.16% cholate as described for Asolectin. After dialysis the vesicles were either stored on ice and assayed as soon as possible or frozen in liquid nitrogen and then thawed on ice before assays were done.

Assays of acetylcholine receptor function

Acetylcholine receptor samples were assayed for equilibrium binding of ^{125}I-labeled α-bungarotoxin in Triton X-100 at room temperature using the DEAE filter disc assay originally described by Schmidt and Raftery [26] as modified [27]. The rate of ^{125}I-labeled α-bungarotoxin binding to membrane bound receptor was measured in

the absence and presence of carbachol in the absence of detergent as described previously [27]. The amount of acetylcholine receptor on the outside was determined by preincubating the vesicles with unlabeled *Naja naja siamensis* α-neurotoxin before carrying out an equilibrium toxin binding assay with ^{125}I-labeled α-bungarotoxin.

The ion permeability control properties of reconstituted acetylcholine receptor were assessed by $^{86}Rb^+$ influx in the presence and absence of carbachol. External cations were separated from trapped ions by the ion exchange procedure of Epstein and Racker [15] using Dowex 50W-X8 resin and disposable 2 ml Isolab columns (QSY). For $^{22}Na^+$ influx, 15 ml PCS were added to the eluate. For $^{86}RB^+$, no scintillation fluid was added. (In the presence of scintillation fluid, a minor contaminant in $^{86}Rb^+$ contributes to a high background. In the absence of scintillant, the counting efficiency is decreased only 50% and the background is essentially zero.) In a typical assay, 50 μl vesicles were mixed with 10 μl of a solution containing 1–2 μCi isotope and 6 mM carbachol (or no carbachol). Influx was allowed to proceed for 10 s and then 50 μl were applied to a column and eluted with 3 ml 175 mM sucrose. The assays were run in triplicate or quadruplicate at room temperature.

To measure the total internal volume of the vesicles aliquots were allowed to incubate for 48 h at 4°C with the radioisotope and then they were passed through the columns. Internal volumes are expressed as nl internal volume per μmol exogenous lipid. Phospholipid content was measured by the method of McClare [28] and over 90% of the lipid phosphorus was recovered after passage of the vesicle through the column.

Sucrose gradient analysis of reconstituted membranes

Duplicate 200 μl aliquots of the reconstituted samples were incubated with sufficient ^{125}I-labeled α-bungarotoxin for 30 min to saturate approx. 5–10% of the binding sites. The labeled toxin was then used as a marker for the presence of acetylcholine receptor. The aliquots were placed either at the top or the bottom of a 5–20% sucrose gradient in buffer A and centrifuged at 60000 rpm for 18–20 h in an SW60 rotor. For samples placed at the bottom, the aliquots were diluted with an equal volume of 60% sucrose before injecting them carefully at the bottom of the tube. Control centrifugations included samples of acetylcholine receptor only (no added lipids) and lipids only. 150-μl fractions were taken from each tube and assayed for ^{125}I cpm or for lipid phosphorus. In some samples containing ^{14}C-labeled lipids, the ^{14}C-labeled lipids were counted directly and no iodinated toxin was added.

Miscellaneous chemicals

Carbamylcholine and cholesterol were obtained from Sigma Chemical Co; $^{22}Na^+$, $^{86}Rb^+$ and ^{125}I-labeled α-bungarotoxin were obtained from New England Nuclear; pure phospholipids were obtained from Avanti.

Results

Extraction and purification of acetylcholine receptor

In order to simplify the interpretation of membrane protein function in a reconstituted system, it is essential to isolate and purify the functional components. In these experiments, acetylcholine receptor was obtained in two ways. First, a highly purified membrane fraction was obtained by a combination of differential and sucrose density gradient centrifugation followed by alkaline extraction of peripheral membrane proteins [23]. Extraction of these membranes with cholate gave a soluble protein fraction containing up to 40% acetylcholine receptor. Second, acetylcholine receptor was purified to homogeneity by an affinity chromatography technique using carboxymethylcholine as the column-bound ligand [24]. The acetylcholine receptor was extracted from partially purified membranes with cholate and all washing and elution steps were carried out in the presence of added phospholipid (DOPC). It has been shown that failure to include lipid results in irreversible loss of acetylcholine receptor ion permeability control properties [29]. The affinity column purified acetylcholine receptor provides a simpler molecular species to analyze, and thus far we have detected no differences between membrane-extracted acetylcholine receptor and purified acetylcholine receptor. For some of the lipid combinations to be described later, only one of the two

kinds of acetylcholine receptor preparation was used.

Table I provides a summary of acetylcholine receptor purification by the affinity chromatography procedure. Typically, 600 g tissue gave 30–50 mg purified acetylcholine receptor. The purity was assessed both by iodinated α-bungarotoxin binding and by SDS-polyacrylamide gel electrophoresis. Fig. 1 shows a scan of the Coomassie brilliant blue staining pattern of purified receptor. The four subunits (α, β, γ, δ) of molecular weight 40, 50, 60 an 65 kDa are the only species present and the stoichiometry ($\alpha_2\beta\gamma\delta$) is typical of pure receptor [30]. The specific activity for toxin binding reported in Table I is also typical of purified acetylcholine receptor (8 nmol/mg protein). In some preparations containing Asolectin, the specific activities were about one-half the expected values, even though the SDS gel patterns appeared normal. We attribute the lower values to interference by the Asolectin during the filtration step of the assay. As observed by Lindstrom et al. [16] the SDS gel patterns appear to provide the most reliable indication of purity in the cholate/Asolectin mixtures.

TABLE I

EXTRACTION AND PURIFICATION OF *TORPEDO CALIFORNICA* ACETYLCHOLINE RECEPTOR

Methods for extraction, purification, and assay are described in Materials and Methods. Results are for a typical preparation starting with 600 g frozen electric tissue from *T. californica*. The lipid used in the column washing step was dioleoylphosphatidylcholine. For six different preparations the yields of pure receptor varied from 22–41%. The specific activities (nmol.mg protein) varied from 7.7–8.4. In four preparations using Asolectin as the lipid, the final specific activities averaged 4.2 nmol/mg.

Stage of purification	Protein		^{125}I-labeled α-toxin binding		
	mg	%	nmol	%	nmol/mg protein
Crude membrane preparation	1550	100	716	100	0.46
1% cholate mixture	1541	99	652	91	0.42
1% cholate supernatant	990	64	626	87	0.63
Purified and reconstituted receptor	32	2	268	37	8.38

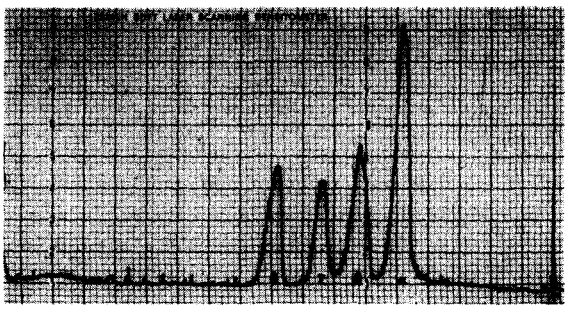

Fig. 1. Scan of the Coomassie brilliant blue staining pattern of purified *Torpedo* acetylcholine receptor after SDS gel electrophoresis on a 1 mM slab gel. The four receptor subunits of 40 (α), 50 (β), 60 (γ), and 65 (δ) kDa are the only polypeptides and the subunit stoichiometry of $\alpha_2\beta\gamma\delta$ is apparent. Acetylcholine receptor was purified as summarized in Table I. The scan was done with a Zeineh soft laser scanner.

Functional properties of reconstituted membranes

The most important functional property of acetylcholine receptor is the opening of a cation-selective ion channel in response to activator binding. We have used carbamylcholine stimulated influx of radioactive cations into membrane vesicles as our principal assay for successful reconstitution of acetylcholine receptor function. If a flux response is obtained there is a guarantee that at least some of the receptors are oriented properly in sealed vesicles and retain both the ligand binding and ion permeability control properties of the receptor. A more difficult (but no less important) aspect of reconstitution is to determine quantitatively the fraction of acetylcholine receptor molecules that are active and contribute to the measured flux response.

A typical 10 s influx response in a reconstituted vesicle preparation is shown in Table II. The vesicles were prepared with purified acetylcholine receptor and Asolectin, a crude soybean lipid mixture. Asolectin was chosen for these experiments because it is the lipid most commonly used in reconstitution studies and it will serve as a references point for the defined lipid studies described later. Carbachol at a concentration of 1 mM greatly increased the influx of $^{86}Rb^+$ into the membranes.

TABLE II

$^{86}Rb^+$ INFLUX INTO RECONSTITUTED *TORPEDO* MEMBRANES

Reconstitued membranes were prepared from purified *Torpedo* acetylcholine receptor and Asolectin as described in Materials and Methods. Vesicles were pre-treated for 10 min with buffer only, 1 mM carbachol, or 1 mM (+)-tubocurare before measuring $^{86}Rb^+$ influx for 10 s at room temperature in the presence or absence of 1 mM carbachol. For the data shown, the final lipid concentration was 20 mg/ml, the acetylcholine receptor concentration was approx. 1 mg/ml and the total $^{86}Rb^+$ cpm applied to the column were $6.2 \cdot 10^6$. In the absence of membranes, the background counts not retained by the ion-exchange column were very low, as shown. Values given are the results of triplicate measurements ± S.E. For six different preparations normalized to the same $^{86}Rb^+$ cpm, the no-carbachol values ranged from 1500 to 4500 cpm and the values with carbachol ranged from 12500 to 31500.

Sample	$^{86}Rb^+$ uptake (cpm)
No membranes	245 ± 87
Membrane + no carbachol	4012 ± 120
Membrane + 1 mM Carbachol	14895 ± 1012
Membrane + 1 mM (+)-tubocurare + 1 mM carbachol	3381 ± 174
Membrane + 1 mM carbachol 10 min preincubation + 1 mM carbachol	4460 ± 513

The effect was completely blocked by (+)-tubocurare and also by prior incubation with 1 mM carbachol, thus demonstrating the expected properties of antagonist blocking and agonist-induced desensitization. For the best preparations, 1 mM carbachol caused a 16-fold increase in the Rb^+ uptake.

Effects of lipid composition on acetylcholine receptor-mediated ion flux responses

In an earlier communication Dalziel et al. [18] showed that cholesterol dramatically increased the flux response when membrane-extracted acetylcholine receptor was reconstituted in the presence of a defined lipid mixture containing phosphatidylethanolamine (PE) and phosphatidylserine (PS) in a 3 : 1 ratio. A more striking cholesterol effect is shown in Table III for reconstituted vesicles prepared with purified acetylcholine receptor and assayed for ion flux by the ion exchange method instead of the filtration procedure used previously [19]. The presence of 50 mol% cholesterol resulted in a 10-fold enhancement of flux response compared to the no-cholesterol control. A representative summary of influx data for vesicles prepared with other lipid mixtures is provided in Table IV. The presence of either PE or PS appeared necessary for a flux response.

TABLE III

EFFECTS OF CHOLESTEROL ON ACETYLCHOLINE RECEPTOR-MEDIATED CATION INFLUX

Reconstituted membranes were prepared from purified acetylcholine receptor and the method for sample preparation and influx assay are described in Materials and Methods. PE is egg phosphatidylethanolamine, PS is bovine brain phosphatidylserine and the ratio is a mole ratio. Response is measured as the difference in cpm between samples with and without 1 mM carbachol (carb.). All assays were done in triplicate at 25°C on non-frozen membranes and the values were all within 5% of each other. The experiment was replicated twice with similar results.

Mol% cholesterol in a PE : PS (3 : 1) mixture	$^{86}Rb^+$ influx (cpm)($\times 10^{-3}$)		
	− Carb.	+ Carb.	response
0	0.7	6.0	5.3
10	0.9	12.9	12.0
25	2.2	18.5	16.3
35	2.9	30.3	27.4
50	3.6	50.1	46.5

In an effort to simplify further the analysis of lipid effects, mixtures consisting of PC and phosphatidic acid were analyzed with and without cholesterol. Phosphatidic acid is a negatively charged lipid and can easily be prepared from PC by phospholipase D hydrolysis. PC/phosphatidic acid mixtures were attractive from a biophysical viewpoint, since the effects of chain length and unsaturation could be tested by taking advantage of the ready availability of many synthetic PC molecules. In contrast, PE and PS molecules are difficult to synthesize, expensive to buy, and available only in a limited number of acyl chains. The effects of PC/phosphatidic acid mixtures on ion flux are summarized in Table V for membranes prepared both from purified and membrane-extracted acetylcholine receptor. As observed for the PE and PS mixtures, the addition of cholesterol

TABLE IV

EFFECT OF DIFFERENT LIPID COMPOSITIONS ON ACETYLCHOLINE RECEPTOR MEDIATED CATION INFLUX

Procedures were as described in legend to Table II. PE, egg phosphatidylethanolamine; PS, bovine phosphatidylserine; PC, dioleoylphosphatidylcholine; chol., cholesterol). All lipid mixtures were used in simultaneous reconstitutions on the same extracted receptor preparation from purified *Torpedo* membranes. Influx responses were measured in quadruplicate and all values were within 10% of the mean. The relative flux responses represent actual results from one preparation. In separate experiments with different lipid combinations, qualitatively similar results were obtained but the relative responses varied. For example, PS/cholesterol mixtures gave a small flux response in some preparations.

Lipid mixture (mol%)				Relative response (%)
PE	PS	PC	Chol.	
56	19	0	25	100
0	19	57	25	47
56	0	19	25	56
0	0	75	25	0
0	75	0	25	0
(Asolectin)				73

had a large effect on the flux amplitude. The most striking effect was the absence of a flux response when DOPC only was used as the lipid. The data also show that the total lipid concentration had a large effect on the measured responses, as expected.

Effects of lipids on acetylcholine receptor binding properties

In parallel with the ion flux studies, the effects of different lipids on the ligand binding properties of the acetylcholine receptor were examined by measuring both equilibrium toxin binding and the rate of toxin binding. The effect of agonists on toxin binding rates has been shown to provide a sensitive indicator of the functional state of the receptor [27]. None of the synthetic lipid mixtures had any differential effect on toxin binding in detergent solution. In addition, none of the lipids altered the effects of carbachol on the rate of binding of toxin in the absence of detergent. Carbachol has been shown to induce a time- and concentration-dependent increase in the binding

TABLE V

ACETYLCHOLINE RECEPTOR ION FLUX RESPONSE IN LIPID MIXTURES CONTAINING DOPC, PHOSPHATIDIC ACID (PA) AND CHOLESTEROL (CHOL)

Membranes were prepared as described in Materials and Methods. Preparations A and C used affinity column purified acetylcholine receptor, whereas preparation B used acetylcholine receptor extracted from highly purified membranes. The lipid/protein ratio was calculated using the known amounts of lipid added to the reconstitution mixture and the protein concentration measured after dialysis. Flux response is expressed as the difference in pmol of Rb^+ taken up by the vesicles per nmol of acetylcholine receptor in the presence and absence of 1 mM carbachol during a 10 s influx assay. Flux responses were measured in quandruplicate and the values given are the means. The standard error was less than 10% for all samples.

Lipid	Preparation	Total lipid concentration (mM)	Lipid protein ratio (w/w)	Flux response
DOPC (100%)	A	11	19	0
	B	27	50	1144
	C	33	84	0
DOPC + PA (75% + 25%)	A	–	–	–
	B	27	42	5616
	C	33	84	14820
DOPC + PA + Chol. (56% + 19% + 25%)	A	11	19	11407
	B	27	42	41616
	C	33	67	41361
DOPC + Chol (75% + 25%)	A	–	–	–
	B	27	39	72
	C	–	–	–

affinity of acetylcholine receptor for agonists [31]. Preincubation of membranes with carbachol results in a transition to a higher affinity state that can be detected as an increased effect of carbachol in slowing down the initial rate of toxin binding. The effect has been exploited to measure both the low- and high-affinity K_d values for carbachol binding [27]. The high-affinity binding sites are characteristic of fully desensitized acetylcholine receptor. Fig. 2 shows that the carbachol-induced binding transition was preserved in reconstituted vesicles containing only DOPC and acetylcholine receptor under conditions in which no flux re-

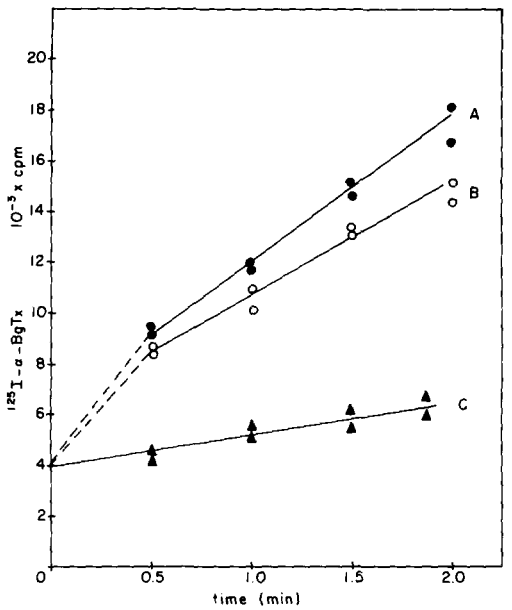

Fig. 2. Effect of agonist (10 μM carbachol) on the initial rate of α-bungarotoxin binding to reconstituted membranes. Membranes containing purified acetylcholine receptor in DOPC were preincubated for 10 min in buffer and then reacted with ^{125}I-labeled α-bungarotoxin (^{125}Iα-BgTx) in detergent-free buffer with or without 10 μM carbachol. The rate of toxin binding was measured by filtering aliquots at various times as described previously [27,18]. (A) No carbachol during preincubation on reaction; (B) no carbachol during preincubation, 10 μM carbachol present during reaction; (C) 10 μM carbachol present during preincubation and reaction.

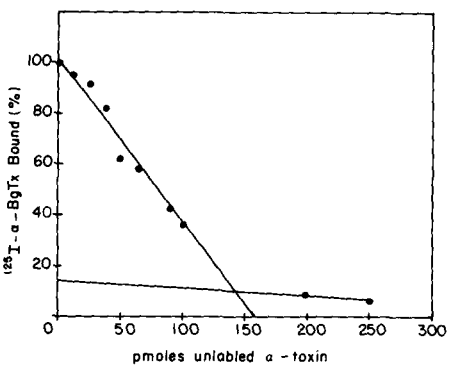

Fig. 3. Sidedness of acetylcholine receptor in reconstituted membranes. Aliquots of reconstituted membranes containing approx. 150 pmol each of α-bungarotoxin binding sites were preincubated with 0–250 pmol of unlabeled *Naja naja siamensis* α-neurotoxin for 30 min in 100 mM NaCl/10 mM Mops (pH 7.4)/1 mg/ml bovine serum albumin for 60 min at room temperature to label exposed binding sites. A standard equilibrium ^{125}I-labeled α-bungarotoxin (^{125}I-α-Bgtx) binding assay was then performed in detergent solution. The amount of labeled toxin bound is plotted against added unlabeled toxin. 100% is taken as the value in the absence of added unlabeled toxin. The break in the curve is due to the presence of inaccessible binding sites in the absence of detergent.

sponse was measurable. Similar binding results were obtained previously [18] and indicate that the lack of a flux response was not due to a loss of binding sites or to a shift to a fully desensitized binding state.

The rate binding data also provide evidence that a significant fraction of the toxin binding sites must be exposed to the external medium, since the assay was done in the absence of detergent and the toxin (M_r 8000) cannot penetrate the vesicles [32]. A more quantitative analysis of exposed acetylcholine receptor sites was made by using increasing amounts of unlabeled *Naja naja siamensis* α-neurotoxin to block external acetylcholine receptor sites prior to a normal equilibrium binding assay with iodinated bungarotoxin. A typical profile is shown in Fig. 3 and the break is taken to represent saturation of external sites. For the data shown, approx. 75% of the binding sites were exposed. Generally, the exposed values have ranged from 75–90% in six different reconstituted preparations. For native *Torpedo* membranes, typical values are 90–100% (Walker and McNamee, unpublished data; see also Refs. 33, 34).

Physical properties of the reconstituted membranes

The results thus far provide a strong indication that lipid composition has a large effect on acetylcholine receptor-mediated ion flux. However, the flux response as measured is very sensitive to the concentration of acetylcholine receptor in a given vesicle, the internal volume of the fluxing vesicle population, and the integrity of the acetylcholine receptor-containing vesicles. For example, the lack of a flux response for acetylcholine receptor in DOPC vesicles could result from improper (or non-existent) incorporation of the protein into the vesicle, a very small internal volume, leaky vesicles, a specific DOPC effect, or some combination of all of the above. Popot et al. [35] have suggested that some lipids might exclude

acetylcholine receptor molecules from the membranes during the reconstitution process.

In order to examine some of these possibilities, reconstituted vesicles were centrifuged through a sucrose density gradient to separate unincorporated protein from lipid vesicles. In some experiments, the reconstituted mixture was placed at the bottom of the centrifuge tube. The movement of the protein up the tube during centrifugation as shown in Fig. 4 provides good evidence that the protein was associated with the lipids and avoids artefactual co-migration of lipids and proteins down the tube when both are placed on top. When acetylcholine receptor alone without added lipid was placed at the bottom of a 5–20% sucrose gradient it remained on the bottom, as expected from its density. For all lipids used, including the non-fluxing DOPC vesicles, greater than 85% of the acetylcholine receptor co-migrated with the lipid peak. Identical results were obtained with both top-loading and bottom-loading of the samples.

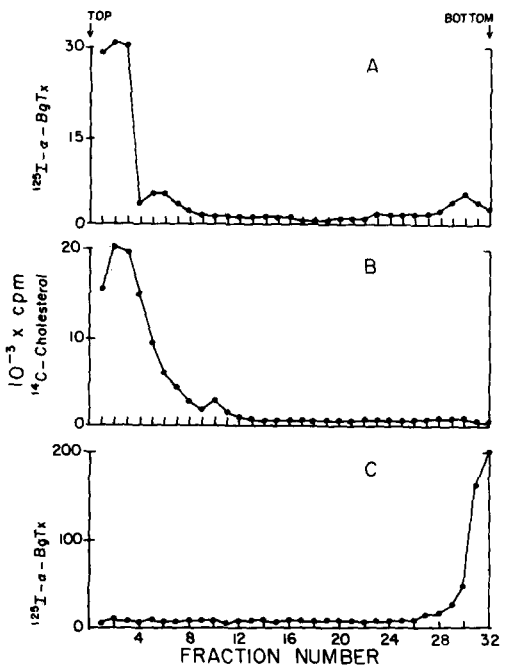

Fig. 4. Analysis of reconstituted membranes on sucrose density gradients. A 200 μl aliquot of a reconstituted sample was carefully injected at the bottom of a 5–20% linear sucrose gradient in buffer A. (A) Acetylcholine receptor in DOPC/phosphatidic acid/cholesterol vesicles incubated with ^{125}I-labeled α-bungarotoxin to label approx. 5% of the available sites; (B) DOPC/phosphatidic acid/cholesterol vesicles with no acetylcholine receptor. [^{14}C]Cholesterol (New England Nuclear) was added before dialysis; (C) acetylcholine receptor alone labeled with ^{125}I-labeled α-bungarotoxin (^{125}I-α-BgTx). The samples were centrifuged for 19 h at 55000 rpm in an SW60 rotor at 4°C. 150-μl fractions were collected from each tube and counted.

The equilibrium internal volume of the vesicles was measured after 48 h of incubation with radioactive cations in order to determine the relative sizes of the vesicles prepared with different lipids. The DOPC vesicles had a very small internal volume, whereas the Asolectin and DOPC/phosphatidic acid/cholesterol vesicles had a significantly larger internal volume (Table VI). For lipid compositions in which the cholesterol concentration was increased while maintaining a constant total lipid concentration, the internal volume increased with increasing cholesterol concentration, but not as dramatically as the flux responses. At the lipid concentrations used in most of these experiments (30 mM), the calculated diameter for a homogeneous vesicle population with an internal volume of 500 nl/μmol would be 400 Å [36].

TABLE VI

EFFECT OF FREEZE-THAW PROCEDURE ON THE INTERNAL VOLUME AND ION FLUX RESPONSE OF RECONSTITUTED MEMBRANES

Reconstituted membranes were prepared from purified acetylcholine receptor as described in Materials and Methods. Lipid-to-protein ratios (w/w) were 50:1 and the total lipid concentration was 33 mM. Internal volumes are expressed as nl solution trapped within the vesicles per μmol total added lipid after 48 h equilibration at 4°C as measured by the amount of $^{86}Rb^+$ that elutes with the vesicle fractions after ion exchange chromatography. Influx response is measured as [$^{86}Rb^+$ cpm(+carbachol) − $^{86}Rb^+$cpm(−carbachol)]/$^{86}Rb^+$cpm(−carbachol) after correction for background cpm in the absence of membranes. Results are the average of triplicate measurements.

Lipid composition	Internal volume (nl/μmol lipid)		Influx response	
	Non Frozen	Frozen	Non Frozen	Frozen
DOPC	87	2445	0	0.91
DOPC/PA/chol. (57:19:25)	398	1259	0.51	3.39
Asolectin	418	1826	16.0	2.26

Previous electron microscopy data has shown that 500 Å is a typical size for Asolectin vesicles [37]. For the most active vesicles used here, the influx response observed in the presence of 1 mM carbachol for 10 s accounted for 60% of the total internal volume.

Effect of freezing on vesicle properties

In earlier experiments [18] it was observed that freezing of the reconstituted vesicles in liquid nitrogen followed by thawing at room temperature increased the retention of the vesicles on Millipore filters. The effect of the freeze-thaw cycle on vesicle flux, internal volume and size was examined for reconstituted membranes with different lipids. In all cases the freeze-thaw cycle increased the internal volume of the vesicles (Table VI). The effect was most dramatic for the DOPC vesicles. After the freeze-thaw cycle, the internal volume of the DOPC vesicles had increased 10-fold and was greater than the volume of the Asolectin and DOPC/phosphatidic acid/cholesterol vesicles. Fusion of pure lipid vesicles after freeze-thaw cycles is well documented [38], and the large increases in internal volume observed here are attributed to vesicle fusion. Repeated freeze-thaw cycles did not change the properties of the vesicles any further. The frozen vesicles appeared to be considerably more heterogeneous than the unfrozen vesicles and showed some evidence of multilamellar structures in preliminary electron microscopy studies. A detailed electron microscopy and gel filtration analysis of the vesicles is in progress. Recent independent studies by Anholt et al. [39] provide good evidence for increased vesicle size after freeze-thaw cycles. The fraction of bungarotoxin binding sites exposed to the external solution was unaltered by the freeze-thaw cycle indicating that no significant amount of acetylcholine receptor was associated with the internal membranes of multilamellar vesicles.

The freeze-thaw procedure had a large effect on dose-response curves for carbachol-stimulated ion flux. For DOPC/phosphatidic acid/cholesterol vesicles, the concentration of carbachol that gave a half-maximum flux response (EC_{50}) was shifted by up to two orders of magnitude to higher concentrations of carbachol following freezing (Fig. 5). In many preparations a smaller shift (5–10-fold)

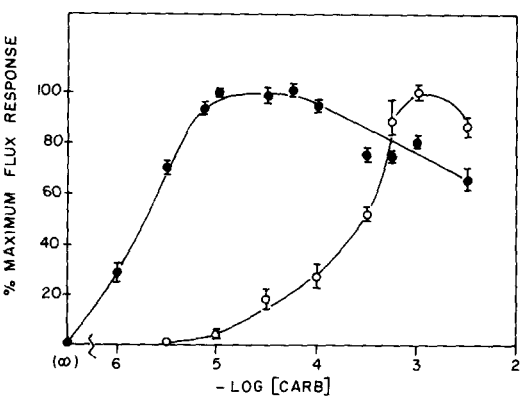

Fig. 5. Dose-response curves for carbachol-stimulated $^{86}Rb^+$ influx into reconstituted vesicles prepared from purified acetylcholine receptor and DOPC/phosphatidic acid/cholesterol. Influx assays were carried out in triplicate as described in Materials and Methods for each of the carbachol concentrations ([CARB]) shown. '∞' represents no carbachol. The results shown for the non-frozen (●) and frozen (○) vesicles are for different preparations of reconstituted vesicles. The actual maximum response in the non-frozen preparation was 15% of the maximum response in the frozen vesicles.

was observed, mainly due to an increase in the EC_{50} for the non-frozen vesicles. The shift reflects the fact that the flux response in non-frozen vesicles is probably limited by the small internal volumes. Thus, apparent saturation occurs at carbachol concentrations well below the concentrations necessary to saturate all the ligand binding sites. Initial rate measurements carried out on Asolectin vesicles containing acetylcholine receptor have clarified the relationship between dose-response curves and acetylcholine receptor activation [40]. The fall off in flux responses at very high carbachol concentrations reflects the onset of a fast desensitization process [40]. Similar dose response curves for non-frozen vesicles under conditions similar to those used here have been observed previously by Lindstrom et al. [16] in Asolectin-acetylcholine receptor vesicles.

The most striking result of the freeze-thaw procedure was the recovery of a flux response in vesicles containing DOPC as the only added lipid. The absence of a flux response in non-frozen vesicles may be attributed, at least in part, to the small size of the vesicles. However, even after freezing, the DOPC vesicles were not as active as

the DOPC/phosphatidic acid/cholesterol and Asolectin vesicles (Table VI).

Discussion

The effects of different lipids on the ion permeability control properties of the acetylcholine receptor can be very dramatic, as illustrated in Table II for cholesterol. However, the internal volume measurements and the freeze-thaw data indicate that the different lipids may be more important in affecting the process of reconstitution than in affecting the function of the acetylcholine receptor itself. When acetylcholine receptor was purified and reconstituted by the cholate dialysis technique in the presence of phosphatidylcholine alone, the resulting vesicles showed no ion permeability control properties although it could be shown that acetylcholine receptor was associated with the lipid vesicles and retained all its specific binding properties. However, after a single freeze-thaw cycle the vesicles showed a 10-fold increase in internal volume and gave a modest ion flux response. Since the size of a flux response measured under the conditions used here (10 s influx time) is limited by the accessible internal volume in the active vesicles [40] the failure to observe a flux response before the freeze-thaw cycle can primarily be attributed to the small size of the DOPC vesicles. The observations reported here are generally consistent with those obtained by Popot et al. [35] except that we always observe acetylcholine receptor association with lipids even when using pure phosphatidylcholine.

Little is known about the molecular process by which lipids and proteins reassociate during detergent removal to form sealed bilayer vesicles, but it is not surprising that cholesterol and negatively charged lipids have a large effect on the process. A crude lipid mixture such as Asolectin often gives the best reconstitution results before freeze-thaw, and most of the reconstitution studies involving acetylcholine receptor have been carried out using both cholate and Asolectin.

It is not known whether the lipid effects on vesicle size and function observed here will be seen with other detergents. Martinez-Carrion and colleagues [41] have used octylglucoside to reconstitute acetylcholine receptor function using extracted lipids from *Torpedo* membranes. They claim not to require added lipids during the purification stages. In contrast, Lindstrom and co-workers [29] showed that octylglucoside irreversibly inhibited channel activity when used in their reconstitution process with Asolectin. It is likely that the combination of particular lipids with particular detergents is critical.

Our demonstration of a flux response in reconstituted vesicles containing only DOPC and pure acetylcholine receptor after the freeze-thaw cycle represents the simplest system yet developed in which all the functional properties of the acetylcholine receptor can be studied. We are preparing to measure the initial rates of the ion influx using quench flow techniques with millisecond times resolution [42]. The initial rate studies will allow us to determine whether the acetylcholine receptor in DOPC vesicles is as quantitatively functional as it is in native *Torpedo* membranes [13,14] and in reconstituted membranes prepared with Asolectin [39]. If the DOPC vesicles are fully functional, they will be ideally suited for biophysical studies of the effects of lipid-protein interactions, phase transitions, bilayer fluidity and bilayer thickness on acetylcholine receptor function.

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