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Reconstitution of the nicotinic acetylcholine receptor using a lipid substitution technique

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The nicotinic acetylcholine receptor was purified by affinity chromatography in the presence of dioleoylphosphatidylcholine (DOPC). A method for replacing the DOPC with other lipids was developed by using detergent solubilization with a large excess of the new lipid followed by sucrose density gradient centrifugation in detergent-free buffers to separate receptor-lipid complexes from excess lipid and detergent. Homogenous complexes of defined lipid composition could be easily prepared and the efficiency of substitution was independent of lipid type. However, the functional properties of the resulting lipid complexes depended on the lipid composition.

Introduction

A central problem in studying the biology of membranes is in defining the role of the constituent lipids [1]. In addition to their structural role, there is now considerable evidence that lipids may modulate membrane function by interacting directly with membrane proteins [2]. In order to investigate lipid–protein interactions, one of the most powerful approaches is to use reconstitution techniques to prepare model systems consisting of purified proteins in a defined lipid environment [3,4]. A particularly promising model system is the reconstituted nicotinic acetylcholine receptor (AChR) of *Torpedo californica* [5,6]. The AChR is an integral membrane protein which transduces the binding of activating ligands into a large increase in the permeability of the postsynaptic membrane to cations [6–8]. The most important properties of AChR include the ability to bind ligands, the coupling of ligand binding to the control of ion channel gating, the ion permeation mechanism, and, in the prolonged presence of agonists, an allosteric state transition in ligand binding which leads to the reversible inactivation of ion permeation [9,10].

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Abbreviations: AChR, acetylcholine receptor; Mops, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DOPC, dioleoylphosphatidylcholine; FAMES, fatty acid methyl esters; ^{125}I-BTX, ^{125}I-labelled monoiodinated α-bungarotoxin; DPPC, dipalmitoylphosphatidylcholine; DPEPC, dipalmitoleylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine. GLC, gas-liquid chromatography.

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Recently, it has become possible to purify and reconstitute AChR into a variety of lipid mixtures with full recovery of functional properties [6,11–13]. Unfortunately, there are still several problems associated with current reconstitution protocols. A particular problem, when detailed biophysical studies are to be made, is the heterogeneous nature of the products of reconstitution, particularly the non-uniform distribution of receptor-containing membranes and their disparate morphology [14–17]. Moreover, current reconstitution protocols are often not suited to biochemical studies in which it is essential to be able to distinguish the effects of different lipids on AChR function from the effects those lipids may have on the reconstitution process [6,13].

In this paper we describe an improved reconstitution technique for the preparation of a homogeneous population of lipid-AChR complexes in various test lipids. The method is adapted from the lipid substitution technique for the preparation of $(Ca^{2+} + Mg^{2+})$-ATPase in defined lipids [18,19]. Briefly, AChR with its associated (i.e., endogenous) lipids is equilibrated with a large excess of test lipid in the presence of the detergent, sodium cholate. Once equilibrated, the mixture is then centrifuged into a detergent-free sucrose density gradient. As the dense micelles of lipid, receptor and detergent sediment, bound cholate diffuses from the micelle and the lipid-substituted complex is reconstituted in a recoverable form.

Materials and Methods

Lipid substitution. Test lipid (5 mg) was dried to a thin film in the bottom of a small glass vial using a gentle stream of dry argon. Residual solvent was removed by vacuum for 1 h. The lipid was then dispersed by adding 125 μl of 2.8% (w/v) sodium cholate in buffer A (10 mM Mops/0.1 M NaCl/0.1 mM EDTA/0.02% (w/v) NaN_3, pH 7.4). The contents of the vial were then closed under argon and clarified by sonication in a water bath above the phase transition temperature of the test lipid. For mixtures of lipids, sonication was performed above the transition temperature of the higher melting component. After cooling the mixture on ice, 125 μl of purified AChR (8 mg/ml; see below) was added at a lipid-to-protein molar ratio (ϕ) of 100 : 1. The sample was allowed to stand at room temperature for 20 min and was then placed on ice for a further 30 min. Following the equilibration period, excess lipid and cholate were separated from AChR-containing material by centrifugation at $320000 \times g_{av}$ (55000 rpm; Beckman SW60 rotor) for 18 h at 4°C on a discontinuous sucrose gradient consisting of 3.1 ml of 25% (w/v) sucrose in buffer A layered on a 800 μl 'cushion' of 70% (w/v) sucrose in buffer A. The substituted complexes were collected from the interface of the 25/70% (w/v) sucrose layers by carefully aspirating the upper layers and manually collecting the opalescent receptor-containing band. In some cases a continuous 0–60% (w/v) sucrose gradient was used. When required, the substituted preparations were dialyzed for 48 h against 2 l of buffer A with three changes to remove traces of residual cholate and then frozen and stored in liquid nitrogen.

Receptor purification. The acetylcholine receptor was purified from frozen *Torpedo californica* electric organ using affinity chromatography exactly as described by Jones et al. [3]. The lipid used in the wash step was dioleoylphosphatidylcholine (DOPC; Avanti Polar Lipids, Birmingham, AL) and typical preparations had a lipid-to-protein molar ratio of 100 : 1. Dialyzed samples of purified AChR were stored in lipid nitrogen prior until use.

Lipid characterization. The extent of lipid substitution was monitored by gas-liquid chromatography (GLC) of the methyl esters of fatty acids derived from the AChR-containing membranes. Lipids, extracted from the substituted membranes (approx. 0.4 mg protein) according to the method of Bligh and Dyer [20], were evaporated to dryness in small (1 ml) GLC vials. The lipid acyl chains were transesterified by adding 100 μl of 14% (w/v) boron trifluoride in methanol [21] and the samples were sealed under argon and heated in a boiling water bath for 20 min. After cooling, the fatty acid methyl esters (FAMES) were twice extracted into ultrapure heptane and aliquots were injected into a GLC apparatus (Packard Instruments) fitted with a 20% diethylene glycol succinate column. The samples were chromatographed at 195°C, using helium gas (40 p.s.i. at a

flow rate of 30 ml/min) as carrier. Elution was monitored with a flame ionization detector. The relative amounts of each ester were determined by quantifying the peak areas.

Analysis of lipid-substituted membranes. The distribution of AChR during lipid substitution was monitored by trace labelling the membranes with ^{125}I-labelled α-bungarotoxin (^{125}I-BTX) as described previously [12,22]. Incorporation of test lipid into receptor-containing membranes was demonstrated by labelling the test lipid with di[^{14}C]palmitoylphosphatidylcholine prior to substitution [22]. Total protein was determined by the Lowry procedure [23] and total phospholipid by the procedure of Yoshida et al. [24].

Functional assays. Irreversible inactivation of AChR by lipid substitution was measured by reconstituting substituted samples into Asolectin vesicles at a high lipid-to-protein molar ratio ($\phi \approx 10\,000$). Briefly, receptor-containing samples were solubilized by adding Asolectin and cholate to the samples to final concentrations of 20 mg/ml and 2% (w/v), respectively. The receptor was then re-integrated into vesicles by extensive dialysis of the solubilized mixture. Carbamylcholine-induced influx of $^{86}Rb^+$ was measured by a 10-s manual assay as described by Walker et al. [25] and Jones et al. [3].

Results

The pattern of events occurring during lipid substitution is schematically illustrated in Fig. 1 and typical results from sucrose density gradient centrifugation are shown in Fig. 2. In these experiments, a mixture containing solubilized AChR in the presence of excess test lipid, e.g., dioleoylphosphatidylcholine, was equilibrated as described in Materials and Methods and centrifuged into a detergent-free sucrose density gradient. By labelling the receptor with a small amount of ^{125}I-BTX, it was possible to monitor the distribution of receptor throughout the gradient. As shown in Fig. 2, the toxin-binding activity corresponding to labelled AChR was distributed as a homogeneous single band at an equilibrium density position corresponding to that for AChR reconstituted at a lipid-to-protein molar ratio (ϕ) of 100. Since any exchange of endogenous for test lipids was

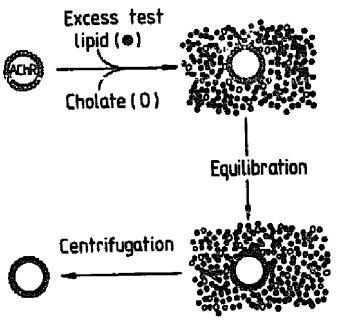

Fig. 1. A schematic representation of the lipid substitution procedure. Receptor-associated lipid is equilibrated with a large excess of test lipid in the presence of the detergent cholate. During centrifugation the receptor and associated test lipid are sedimented, while cholate and excess test lipid remain at the top of the gradient.

expected to lead to incorporation of the test lipid into receptor-associated lipids, the fate of the test lipid was monitored by incorporating a small amount of radiolabelled lipid into the equilibration mixture prior to centrifugation. The results are also shown in Fig. 2 and clearly demonstrate the presence of two pools of lipids: a large pool containing radioactivity corresponding to lipid at the top of the gradient, and a smaller pool of labelled lipid corresponding to test lipid incorporated into the receptor-containing band. Cholate was retained along with excess lipids at the top of the gradient and the levels of cholate in the substituted complexes appeared to be negligible.

Substitution of receptor-associated lipid for test lipids was readily shown by GLC of the fatty acid methyl esters corresponding to those lipids associated with AChR. GLC analysis of fatty acid methyl esters derived from AChR-containing membranes before and after substitution by different test lipids is shown in Fig. 3. When the starting material was AChR reconstituted with DOPC (DOPC-AChR) at a molar ratio of 100, the GLC profiles revealed a single peak with a retention time corresponding to the expected 18 : 1 fatty acid methyl esters (Fig. 3a). However, after equilibration of DOPC-AChR with test lipids, such as DPEPC or DSPC (Fig. 3b and c, respectively) and isolation of substituted complexes by sucrose density gradient centrifugation, GLC analysis of the fatty acid methyl esters

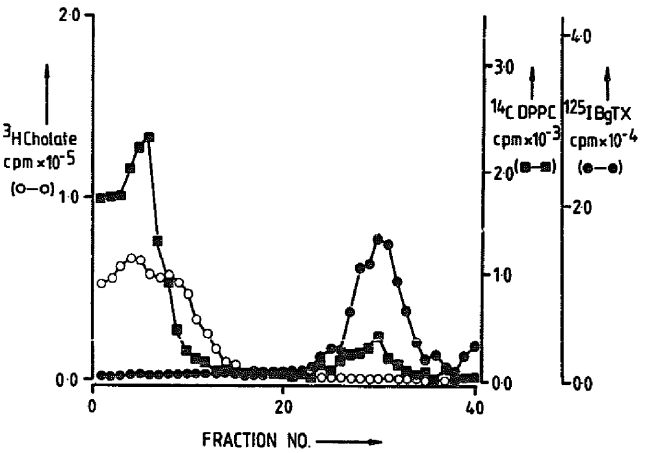

Fig. 2. Analysis of the lipid substitution protocol for AChR using continuous sucrose density gradient centrifugation. DOPC-AChR was equilibrated with a test lipid, DPPC, as outlined in Materials and Methods. Following equilibration, the mixture was applied to a detergent-free 0–60% (w/v) continuous sucrose gradient in buffer A. Following centrifugation, at 320000 × g_{av} for 22 h at 4 °C, the gradients were fractionated manually. The distributions of lipid, cholate and AChR were determined by trace labelling the initial equilibration mixture with [C^{14}C]DPPC (■), [^{3}H]cholate (○) or I^{125}I-labelled α-bungarotoxin (^{125}I-BgTX) (●) in three separate experiments.

of the substituted complexes revealed that at least 90% of the original lipid, DOPC, could be replaced by test lipid (Fig. 3 and Table I). Following a second substitution step the starting lipid could be completely replaced by test lipid.

Results obtained by GLC analysis for a variety of test lipids differing with respect to chain length, degree of saturation of the acyl chains, or lipid headgroup also showed that the extent of substitution was largely independent of the nature of the test lipid (Table I). The agreement between the extent of substitution determined experimentally and that calculated by assuming complete equilibration of endogenous and test lipid pool was good. For example, following substitution of DOPC-AChR by an equimolar mixture of two

TABLE I

SUBSTITUTION OF AChR BY DIFFERENT TEST LIPIDS

Values are given as means ± S.D., where, appropriate, and the numbers of samples tested are shown in parentheses. The position and *cis* or *trans* nature of the double bond are given as Δ, 9*c* and *t*, respectively. DMPC/DPPC refers to an equimolar mixture of the two lipids. The total value of 91.0% substitution by DMPC and DPPC was 45.0 and 46.0 for each lipid, respectively. Lipid-substituted complexes were prepared using the test lipids as described in Materials and Methods.

Test lipid	Acyl chain	Recovery of protein on gradient (%)	Lipid-to-protein molar ratio (φ)	Substitution (%)
DMPC	14:0	81.0 ± 12.0 (7)	93.0 ± 47.0 (8)	93.0
DPPC	16:0	73.0 ± 9.0 (9)	75.0 ± 38.0 (9)	91.0
DSPC	18:0	84.0 ± 8.0 (4)	150.0 (2)	96.0
DPEPC	16:1Δ9*t*	81.0 ± 16.0 (4)	166.0 (2)	94.0
DEPC	18:1Δ9*t*	70.0 (2)	108.0 ± 7.0 (3)	100.0
DOPC	18:1Δ9*c*	74.0 ± 18.0 (4)	137.0 ± 36.0 (4)	100.0
DLPC	18:2Δ9, 11*c*	66.0 ± 15.0 (6)	146.0 ± 32.0 (7)	89.0
DMPC/DPPC	14:0/16:0	86.0 (2)	58.0 (2)	91.0

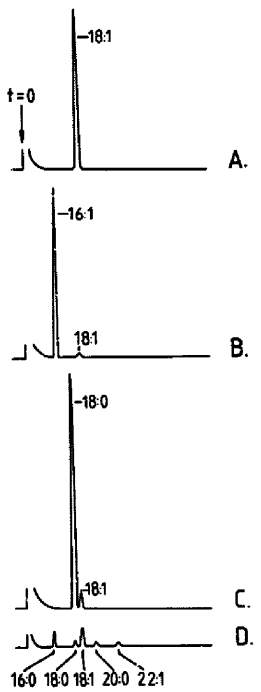

Fig. 3. Exchange of receptor-associated lipids for test lipids determined by GLC analysis of fatty acids extracted from the lipid-substituted complexes. Following lipid substitution membrane lipids were extracted, transesterified and the fatty acid methyl esters were analyzed by GLC (see Materials and Methods). The GLC profiles correspond to lipids derived from AChR substituted with the following test lipids (B) DPEPC and (C) DSPC. A and D show lipids from the starting material DOPC-AChR and a series of standards, respectively.

saturated lipids, DMPC and DPPC, each test lipid in the substituted complex was present in a 1 : 1 molar ratio. In addition, Table I shows that approx. 70% of the receptor protein initially applied to the gradient could be recovered as the substituted complex and the extent of recovery of protein was independent of the nature of the test lipid.

A more marked dependence on the nature of the test lipid was found for the lipid-to-protein ratio of the substituted complexes. Previous results showed that the lipid concentration of the receptor complexes prepared on centrifugation into detergent-free sucrose density gradient depended on the concentration of detergent used to solubilize the membranes [22]. By raising the concentration of detergent in the initial equilibration mixture, it was possible to lower the lipid content of the substituted complexes. By using the concentration of cholate that gives a molar ratio of 100 when DOPC is used (1.4% (w/v) cholate) it was possible to examine the effect of the nature of the test lipid on the lipid concentration of the substituted complexes. From the results (Table I) it is apparent that there is some variability in the molar ratios obtained with the different test lipids. Although some variability can be attributed to errors in measuring the protein and/or the lipid concentration, the greatest discrepancies between the measured and the expected value of 100 : 1 was found with test lipids prone to phase separation, most notably those containing saturated acyl chains.

Although the lipid substitution technique allows the preparation of essentially pure complexes of AChR in defined lipid environments, it is important to show that differences in the functional properties of the AChR in various test lipids truly reflect the effects of those lipids rather than irreversible effects on protein function experienced during lipid substitution.

Unfortunately, the complexes prepared by lipid substitution are at a low lipid-to-protein ratio and form bilayer sheets [22] and thus it was not possible to measure directly those receptor functions associated with ion channel activity. However, in previous studies it has been possible to test for any irreversible inactivation of AChR by re-reconstituting the AChR back into Asolectin – a lipid mixture previously shown to support receptor function fully [5,6,11,12]. By re-reconstituting substituted AChR into Asolectin vesicles, it was possible to examine whether the ion flux properties of the substituted receptor were damaged during substitution. As illustrated in Table II, the specific ion flux activities of the receptor complexes were similar for all the substituted complexes tested, being typically between $1 \cdot 10^{-9}$ and $3 \cdot 10^{-9}$ mol^{-1}. Although discrepancies were seen between samples, these were in fact quite minor compared to the complete failure to detect any ion translocation in inactive samples. The flux assay used here is designed to provide an all or nothing indication of activity. However, it is quite sensitive to pro-

TABLE II

ION PERMEABILITY PROPERTIES OF LIPID-SUBSTITUTED AChR RECONSTITUTED INTO ASOLECTIN

Values are given as means ± S.D. where appropriate and numbers of samples tested are given in parentheses. Samples marked (2X) were substituted twice. The spec. act. of Asolectin samples which have not been lipid substituted is typically around $3.0 \cdot 10^{-9}$ mol^{-1}. Lipid-substituted complexes were solubilized by adding cholate and Asolectin to give a final concentration of 2% (w/v) and 20 mg/ml, respectively. Dialysis and ion flux assays are referenced in Materials and Methods.

Lipid	Flux volume (l) $\times 10^{-9}$	Internal volume (l) $\times 10^{-9}$	Spec. act. (mol^{-1}) $\times 10^{-9}$
DMPC	2.60 ± 1.00 (3)	33.00 ± 6.40 (3)	2.32 ± 0.50 (3)
DPPC	2.20 ± 0.70 (3)	48.00 ± 18.00 (3)	0.76 ± 0.08 (3)
DSPC	1.60 (2)	23.40 (2)	0.80 (2)
DPEPC	4.11 ± 0.70 (4)	47.10 ± 15.2 (4)	2.37 ± 1.80 (4)
DEPC	2.70 ± 0.80 (3)	39.20 ± 3.80 (3)	1.96 ± 0.60 (3)
DOPC	1.25 ± 0.45 (3)	16.60 ± 5.40 (3)	3.32 ± 1.30 (3)
DLPC	1.49 (2)	16.10 (2)	3.04 (2)
DMPS	1.13 (2)	10.70 (2)	3.38 (2)
DPEPC (2X)	5.27 (2)	49.50 (2)	1.50 (2)
DEPC (2X)	1.90 (2)	42.30 (2)	1.30 (2)

gressive inactivation of receptors as demonstrated by Jones et al. [22].

A less direct measure of receptor function is to measure the ability of the receptor to undergo allosteric state transitions in agonist binding affinity thought to accompany the desensitization process [10]. Such transitions are, in fact, quite sensitive to the lipid environment of the AChR and have the advantage that they can be applied to the receptor prepared at low lipid-to-protein ratios [16]. To measure the allosteric transition a toxin rate binding assay, which relies on the competitive binding of α-bungarotoxin and activating ligands, was used. The ability of agonists to reduce the rate of association of toxin to AChR in a state of high agonist affinity is greater than in a low-affinity state. Here, the rate of association of toxin to AChR was measured under three conditions. First, a pseudo-first-order rate constant k_{max} corresponding to the maximum rate of toxin association with AChR was determined. Second, toxin and competing agonist were added to the AChR simultaneously and the rate of toxin-receptor association was measured. The co-incubation

TABLE III

α-BUNGAROTOXIN-BINDING RATE CONSTANTS FOR LIPID-SUBSTITUTED AChR MEMBRANES

Values are given as means ± S.D., where appropriate, and the numbers of samples tested are given in parentheses. Samples marked (2X) were substituted twice. Rate constants for ^{125}I-BGT were measured in the absence of carbamylcholine (k_{max}), during co-incubation with 5 μM carbamylcholine (k_{co}) or after preincubation for 10 min at room temperature with 5 μM carbamylcholine. The AChR concentration was 3 nM and the ^{125}I-BGT concentration was 30 nM.

Lipid	k_{max} (s^{-1}) $\times 10^3$	k_{co} (s^{-1}) $\times 10^3$	k_{pre} (s^{-1}) $\times 10^3$	Transition
DMPC	5.15 ± 1.00 (4)	1.84 ± 0.36 (4)	2.61 ± 0.62 (4)	−
DPPC	4.50 ± 1.10 (3)	3.30 ± 0.40 (3)	2.60 ± 0.40 (3)	+
DPEPC	4.61 ± 0.40 (3)	3.73 ± 0.38 (3)	2.15 ± 0.32 (3)	+
DEPC	7.35 ± 0.22 (3)	5.56 ± 0.77 (3)	3.62 ± 0.76 (3)	+
DOPC	4.70 ± 0.24 (3)	2.10 ± 0.24 (3)	1.03 ± 0.03 (3)	+
DLPC	4.30 ± 1.00 (4)	2.98 ± 0.03 (3)	2.34 ± 0.76 (3)	−
DMPS	4.90 ± 1.10 (4)	2.02 ± 0.28 (4)	2.31 ± 0.39 (4)	−
DPEPC (2X)	5.14 (2)	4.11 (2)	2.48 (2)	+
DEPC (2X)	7.13 (2)	5.55 (2)	4.38 (2)	+

pseudo-first-order rate constant k_{co} corresponds to activating ligands binding to the low-affinity AChR state. Third, the receptor was pre-incubated with agonist and the rate of association of toxin with the receptor was then determined. During preincubation with activating ligands, receptors undergoing the allosteric state transition shift from a state of low to high agonist affinity. The ability of activating ligand to compete with toxin is enhanced and a pseudo-first-order rate constant k_{pre} corresponding to binding to high-affinity AChR is measured. Consequently, for those receptors able to undergo the affinity state transition, k_{co} will lie between the upper limit, k_{max}, and the lower limit, k_{pre}. As shown in Table III, the affinity transition was quite sensitive to the type of lipid in the substituted complex. Although some lipids were unable to support the affinity transition, this effect was not due to irreversible receptor inactivation, since the transitions were recovered simply by substituting the AChR back into a lipid supporting the transition.

Discussion

In this paper we have provided evidence illustrating the suitability of the substitution technique in preparing AChR in a defined lipid environment. The lipid substitution procedure depends on two major factors. First, complete equilibration of the test and endogenous lipid pools and second, the ability to isolate the AChR-test lipid complexes using sucrose gradient centrifugation. Since exchange of test lipid and endogenous lipid pools is very slow in the absence of detergent, it seems likely that the role of cholate is to act as an inert equilibrating agent, as suggested previously [18]. Although we have not conducted a detailed investigation it may also be possible to employ other detergents.

In common with many other successful reconstitution techniques, the lipid substitution technique is conservative, since at no time is the protein delipidated prior to reconstitution into test lipid. A clear advantage of the lipid-substitution procedure is the ease with which a fairly homogeneous population of AChR-lipid complexes can be prepared. Additionally, simply by adjusting the concentration of detergent in the initial equilibration mixture, it is possible to prepare substituted complexes at the desired lipid-to-protein ratio.

In comparison with most other reconstitution procedures, the lipid substitution procedure is both rapid and convenient, being well suited to the preparation of AChR in a variety of test lipids particularly when only small amounts of protein (approx. 1 mg) are required.

Our observations with AChR agree well with those made by other workers for the preparation of $(Na^+ + K^+)$-ATPase [25], $(Ca^{2+} + Mg^{2+})$ ATPase [18,19] and butyrate dehydrogenase [27], by the lipid substitution method. It therefore seems likely that lipid substitution conforms to the scheme outlined in Fig. 1 and may be applicable to the reconstitution of many other membrane proteins.

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