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Biophysical and Ion Channel Functional Characterization of the *Torpedo californica* Nicotinic Acetylcholine Receptor in Varying Detergent–Lipid Environments

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Abstract The nicotinic acetylcholine receptor (nAChR) of *Torpedo* electric rays has been extensively characterized over the last three decades. However, high-resolution structural studies have been hampered by the lack of mechanistic molecular models that describe how detergents influence membrane protein stability and function. Furthermore, elucidation of the dynamic detergent–lipid–protein interactions of solubilized membrane proteins is a largely unexplored research field. This study examines the effects of nine detergents on: (1) nAChR-lipid composition (gas chromatography with flame ionization; GC-FID and/or mass selective detectors; GC-MSD), (2) stability and aggregation state (analytical size exclusion chromatography; A-SEC and electron microscopy; EM) and (3) ion channel function (planar lipid bilayers). Detergent solubilization of nAChR-enriched membranes did not result in significant native lipid depletion or destabilization. Upon purification, native lipid depletion occurred in all detergents, with lipid-analogue detergents CHAPS {(3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate}, FC-12 (*n*-dodecylphosphocholine) and sodium cholate (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) maintaining stability and supporting ion channel function, and non-lipid-analogue detergents Cymal-6 (6-cyclohexyl-1-hexyl-β-D-maltoside), DDM (*n*-dodecyl-β-D-maltopyranoside), LDAO (lauryldimethylamine-*N*-oxide) and OG (*n*-octyl-β-d-glucopyranoside) decreasing stability and significantly reducing or completely suppressing ion channel function. Anapoe-$C_{12}E_9$ (polyoxyethylene-[9]-dodecyl ether) and BigCHAP (*N,N'*-bis-[3-d-gluconamidopropyl] cholamide) retained residual amounts of native lipid, maintaining moderate stability and ion channel function compared to lipid-analogue detergents. Therefore, the nAChR can be stable and functional in lipid-analogue detergents or in detergents that retain moderate amounts of residual native lipids, but not in non-lipid-analogue detergents.

Keywords nAChR · Detergent · Ion channel function · Lipid · Planar lipid bilayer · Biophysical characterization

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Introduction

The nicotinic acetylcholine receptor (nAChR) is an essential component in the cholinergic pathways that regulate and control synaptic transmission in brain and muscle tissues. It is a member of the cysteine loop (Cys-loop) or ligand-gated ion channel (LGIC) family of receptors, which includes the neuronal and muscle-type nicotinic receptors, glycine receptor (Gly-R), γ-aminobutyric acid type-A and type-C receptors ($GABA_A$ and $GABA_C$) receptors, 5-hydroxytryptamine type-3 ($5\text{-}HT_3$), as well as the invertebrate glutamate and histidine receptors (Karlin

2002). nAChRs from *Torpedo* electric rays are five-subunit integral membrane protein complexes with a stoichiometry of $\alpha_2\beta\gamma\delta$ (two α-subunits, one β-subunit, one γ-subunit and one δ-subunit) in a pentameric arrangement, and an internal cation-selective ion channel at the center. Each subunit has four transmembrane segments (M1–M4), and the amino- and carboxy-terminal domains are located extracellularly (Karlin and Akabas 1995). In vertebrate neuromuscular junctions, binding of the endogenous neurotransmitter acetylcholine (ACh) initiates membrane depolarization, generating an action potential that travels across the membrane and ultimately results in the transmission of nerve impulses.

Despite extensive study over the past 30 years, there have been no successful high-resolution structures of the complete nAChR. Recent studies have yielded a three-dimensional nAChR EM structure using native *Torpedo marmorata* membranes (Miyazawa et al. 2003; Unwin 2005), and an X-ray crystal structure of the recombinant mouse α-1 nAChR extracellular domain (Dellisanti et al. 2007). More recently, the crystal structure of a prokaryotic LGIC from *Erwinia chrysanthemi* at 3.3 Å resolution has been reported (Hilf and Dutzler 2008). Similar high-resolution structural data of the intact receptor in the detergent-solubilized state has remained elusive, partly due to a lack of understanding about dynamic detergent–protein–lipid interactions. Previously-published *Torpedo* nAChR structural studies have generally not analyzed the effects of detergent solubilization and purification on *Torpedo* membrane lipid components prior to crystallization trials (Hertling-Jaweed et al. 1988), and how lipid depletion could affect nAChR function and stability. Most nAChR detergent–lipid–protein interaction data have been obtained from receptors prepared from native or sodium cholate–solubilized *Torpedo* membranes that were either: (1) reconstituted into native lipid vesicles through detergent dialysis, or (2) reconstituted into lipid vesicles at defined compositions, by exchanging native lipids for specific exogenous lipids during affinity chromatography purification, followed by detergent dialysis.

Previous studies on the effects of lipids on receptor ion channel function suggests that ligand binding is independent of lipid composition, but nAChR function requires the presence of both neutral and negatively charged lipids (Fong and McNamee 1986). Fourier transform infrared spectroscopy (FT-IR) and labeling studies with [^{125}I]-TID {3-(trifluoro-methyl)-3-(m-[^{125}I]iodophenyl)diazirine}, a photoreactive probe, suggest that the effect of negatively charged phospholipids is synergistic rather than additive (da Costa et al. 2002; Hamouda et al. 2006). Other studies have suggested that the total phospholipid amount required to support nAChR function is $\sim$45 moles of lipid per-mole of receptor, and that complete loss of ion channel function occurred if this ratio fell below $\sim$20 moles of lipid per-mole of receptor (Jones et al. 1988).

Previous analysis of the effects of detergents on nAChR ion channel function showed that sodium cholate and CHAPS could support ion channel flux, but similar experiments with OG remain controversial. Both sodium cholate–solubilized native *Torpedo* membranes reconstituted in soybean lipid vesicles (Anholt et al. 1981), and affinity-purified nAChR solubilized in sodium cholate and reconstituted in soybean, asolectin or exogenous lipid vesicles (Walker et al. 1982; Fong and McNamee 1986; Jones et al. 1988) showed ion channel flux. Affinity-purified nAChRs solubilized in CHAPS and reconstituted in soybean phospholipid and cholesterol vesicles (Schürholz et al. 1992) were also capable of supporting ion channel flux. However, some studies using OG show nAChR ion channel flux for OG-solubilized, affinity-purified nAChR reconstituted in native lipid vesicles (Paraschos et al. 1982), while others show inhibition of ion channel flux for OG-solubilized native *Torpedo* membranes reconstituted in soybean lipid vesicles (Anholt et al. 1981). Receptor ion channel function has also been probed using planar lipid bilayers in native *Torpedo* membranes (Schindler and Quast 1980), which showed electrophysiological parameters similar to detergent-solubilized and Con-A (agarose-linked Concavalin-A) purified receptor in sodium cholate (Nelson et al. 1980), or α-cobratoxin-purified nAChR solubilized in Triton X-100 {α-[4-(1,1,3,3-Tetramethylbutyl)phenyl]-ω-hydroxy-poly(oxy-1,2-ethanediyl)} exchanged into Tween-80 (polyoxyethylene(80)sorbitan monolaureate) during purification (Boheim et al. 1981), using similar planar bilayer techniques.

Given the many variations in receptor preparation protocols in these experiments—type of detergent, whether the receptor is detergent-solubilized or affinity-purified, and the type of lipid the receptor is reconstituted into—it is difficult to develop a clear understanding of the most favorable detergent–lipid combination and purification methodology for preserving nAChR ion channel function. In this study, we report systematic attempts to understand how detergent type, affinity purification and associated lipids affect nAChR stability and function. We use a battery of already proven techniques to assay nAChR lipid composition, ion channel function and stability as well as aggregation state, including planar lipid bilayers, EM, GC-FID, GC-MSD and A-SEC. We identify desirable detergent families for nAChR solubilization and purification in order to routinely obtain stable and functional purified nAChR for future biophysical and structural studies seeking to advance our current understanding of the nAChR.

Materials and Methods

Crude Membrane Isolation, Detergent Solubilization and nAChR Ligand-Affinity Purification

Sucrose gradient isolation of nAChR-enriched crude membrane fractions was performed as described (Ochoa et al 1983), fractioning the gradient from the bottom up for immediate use, or freezing at −20°C. Membranes were diluted in a 1:3 ratio with dialysis buffer-1 (DB-1): 10 mM MOPS (3-(*N*-morpholino)propanesulfonic acid, pH = 7.4), 100 mM NaCl, 0.1 mM EDTA and 0.02% NaN_3. Membrane solubilization was carried out for 1 h at 4°C with a 1–4% final concentration of each detergent (Table 1), followed by high-speed ultracentrifugation for 1 h and application of the supernatant to a 1.5 × 15 cm Econo-column (Bio-Rad Laboratories, Hercules, CA) containing ~12.5 ml bromoacetylcholine affinity resin, prepared as described (Bhushan and McNamee 1990). The column was washed with ~10 times the undiluted crude membrane volume that was originally applied to the column (5 ml of crude membranes = 50 ml of wash buffer), using DB-1 buffer supplemented with the detergent of choice at 1.5 times the critical micelle concentration (CMC) (Table 1). The nAChR was eluted with ~50 ml of wash buffer supplemented with 90 mg of carbamylcholine chloride (Carb; Sigma, St. Louis, MO) and 292 mg of NaCl, concentrated to ~2.5 ml and applied to a PD-10 desalting column (GE Healthcare, Uppsala, Sweden) to remove the Carb ligand. The receptor was eluted from the desalting column with ~5 ml of wash buffer and concentrated to ~125–250 µl before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and BCA™ protein concentration assay (Pierce Biotechnology, Rockford, IL).

Sample Preparation for A-SEC

Approximately 15–25 µl of purified nAChR was reacted with α-bungarotoxin (α-BTX)-Alexa Fluor 488 antagonist (Invitrogen-Molecular Probes, Eugene, OR) in a 2.5:1 α-BTX:nAChR molar ratio for ~2 h at 4°C. 5–10 µl of the complex were injected into an Ultimate-3000 chromatography system (Dionex, Sunnyvale, CA) with a KN-803 analytical column (Shodex, Kawasaki, Japan) in a mobile phase of 20 mM HEPES [4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, pH 7.5], 200 mM NaCl and 0.09% DDM.

Sample Preparation for EM

Affinity-purified nAChR (1–2 µl) was applied to the surface of glow-discharged, carbon-coated 400-mesh cooper grids. After 1 min, the grids were sequentially washed through ten (10) ~200 µl distilled, deionized water drops sitting on the surface of a sheet of parafilm. A 0.5–1 µl drop of 1% uranyl acetate solution was then applied to the grid and allowed to sit for 1 min, before removing the excess liquid by applying filter paper to the edge of the grid. Grids were imaged using a Tecnai F20 Twin transmission EM (FEI, Hillsboro, OR) equipped with a 4 K × 4 K charge-coupled device camera, operating at an accelerating voltage of 120 keV. Grid loading and unloading were performed manually or using a robotic system (Potter et al. 2004), and images were acquired using the Leginon software (Suloway et al. 2005). Low magnification imaging was used to identify up to nine (9) intact grid squares, either by manual inspection or using automated procedures (Cheng et al. 2007). After automated focusing, two (2) to four (4) images per square were acquired at a magnification of 50,000×, with the camera binned by four (4; pixel size 0.652 nm), using an electron dose of 3–5 electrons/Å.

Sample Preparation, Analysis and Data Processing for Planar Lipid Bilayer Assays

Affinity-purified nAChR detergent micelles were coated with PMAL-C8 [poly(maleic anhydride-alt-1-decene substituted with 3-(dimethylamino)propylamine; Anatrace, Maumee, OH] as described (Martínez et al. 2002). A BC-525D bilayer clamp amplifier (Warner Instruments, Hamden, CT), connected to an 8-pole low-pass Bessel filter and a computer through a Digidata 1322-A interface (Molecular Devices, Sunnyvale, CA), was used to probe ion channel function using the following procedure. A mixture of brain L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL) and cholesterol (Anatrace) on a 3:1:1 ratio at ~75 mg/ml in chloroform was prepared, evaporated under nitrogen and resuspended in decane at the same concentration. A 1:1 dilution of this mixture was applied to a 200-µm aperture in a cup-shaped Teflon chamber and allowed to air dry before reopening the aperture and fitting into the rear cavity of a rectangular plastic holder, containing two electrode apertures, which were filled with 1 M KCl. Both the Teflon chamber and the front plastic holder cavity were filled with ~1 ml of bilayer buffer solution: 10 mM HEPES (pH 7.2), 1 mM $MgCl_2$ and 150 mM NaI. Salt bridges filled with 1 M KCl and 2% agarose were used to connect the electrodes to both chambers in the plastic holder, and the Teflon chamber aperture was re-coated with the lipid mixture until a seal was detected (membrane capacitance = 90–170 pA). Approximately 1–25 µg of PMAL-coated nAChR vesicles were added to the front plastic chamber and stirred for ~15 min, verifying protein incorporation through a ~5–10 pA change in membrane

Table 1 Physical properties of detergents used for nAChR biophysical characterization

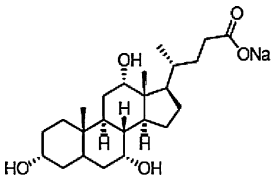
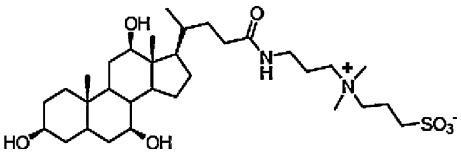
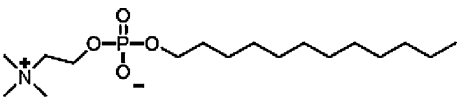
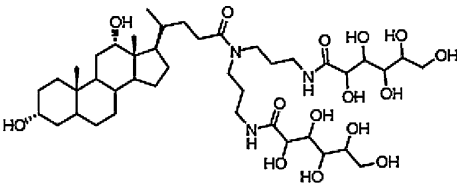
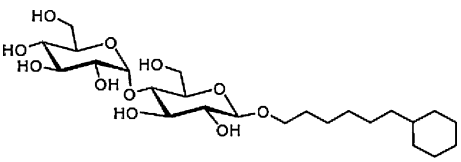
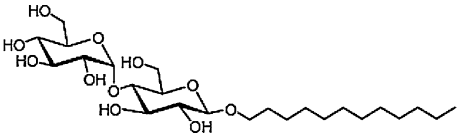
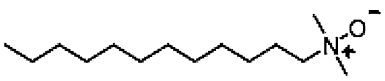
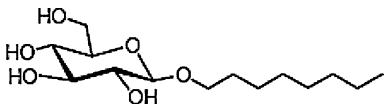
Name	CMC (mM)	Aggregation number	Solubilization concentration (mM)	Affinity column DB-1 wash buffer concentration (1.5 × CMC [mM])	Classification based on experimental results	Structure
Sodium cholate	14	~2–4.8	46.4 (2%)	21	Lipid-analog	
CHAPS	8	10	32.5 (2%)	12	Lipid-analog	
FC-12	1.5	–	28.4 (1%)	2.25	Lipid-analog	
Anapoe-$C_{12}E_9$	0.05	–	17.2 (1%)	0.075	Retained residual amounts of native lipids	$CH_3(CH_2)_{11}O(CH_2CH_2O)_gH$
BigCHAP	2.9	10	45.6 (4%)	4.35	Retained residual amounts of native lipids	
Cymal-6	0.56	63	19.7 (1%)	0.84	Non-lipid-analog	
DDM	0.17	~78–149	19.6 (1%)	0.26	Non-lipid-analog	
LDAO	1–2	–	43.6 (1%)	3	Non-lipid-analog	

Table 1 continued

Name	CMC (mM)	Aggregation number	Solubilization concentration (mM)	Affinity column DB-1 wash buffer concentration (1.5 × CMC [mM])	Classification based on experimental results	Structure
OG	18–20	~27–100	68.4 (2%)	35	Non-lipid-analog	

Classification, physical properties, including critical micelle concentration (CMC), aggregation number, solubilization and affinity column DB-1 wash buffer concentration and structures for each individual detergent

capacitance. Stirring was stopped for data recording, while the membrane potential was gradually increased to +70 mV for 1 min and reversed to −70 mV for 1 min to record a baseline signal with protein and no agonist (Fig. 1a). The membrane potential was turned off before adding 0.5 μM Carb, gradually increasing to +70 mV for 2–5 min or until steady currents were observed, at which point the potential was reversed to −70 mV for the same time frame, continuing these cycles until the membrane broke. Clampfit 10.0 software (Molecular Devices) was used to analyze file segments of 5–10 min, manually searching for 100–1,000 ion channel opening–closing events, which were integrated and processed with a computer, generating mean open channel current and mean open channel time histograms (supplementary materials) from which values for these parameters were obtained for each detergent.

Total Phospholipid and Cholesterol Analysis

Sucrose gradient detergent-solubilized membranes or affinity-purified nAChR samples were subjected to lipid extractions (Bligh and Dyer 1959) containing butylated hydroxytoluene (BHT; 2×10^{-5} M), followed by 3.5 h of reflux with MeOH/HCl for phospholipid hydrolysis. Samples were extracted with petroleum ether (PE), dried under nitrogen and dissolved in 10% MeOH/diethyl ether (DE) for methylation using 0.5 ml of diazomethane. After 20 min, the reaction mixture was extracted with PE and applied to rhodamine 6-G–stained silica gel thin-layer chromatography plates with PE:DE (98:2 v/v) as the solvent system, excising cholesterol and fatty acid methyl ester (FAME) bands for further analysis. Cholesterol was analyzed with the AmplexRed cholesterol assay (Invitrogen, Carlsbad, CA), as described in the manufacturer's instructions. Isolated FAMEs were analyzed with Hewlett-Packard 5890 series II GCs with FID or MSD (5972A) detectors, equipped with a Supelco SPB-5, 30 m × 0.25 mm i.d. column (Sigma-Aldrich, St. Louis, MO). 1 μl of sample in *n*-hexane was injected manually (injector temperature 250°C, oven temperature 130°C), and after 5 min, a 3°C per-min ramp was applied to 200°C (1-min hold), resuming the ramp to 220°C with a 10-min hold as the final step. FAME quantitation was performed with an internal standard of known concentration absent from the lipids in the *Torpedo* tissue ($20{:}1^{\Delta 11}$), dividing the total FAME by two (2) and using appropriate control samples to eliminate trace amounts of contaminants found in all solvents tested. Total phospholipid and cholesterol amounts for both detergent-solubilized sucrose gradient membranes and affinity-purified nAChR samples were normalized using protein concentration values in mg/ml obtained from BCA assay measurements.

Results

Quantitation of Residual Native Lipids Following Detergent Solubilization and nAChR Affinity Purification

In order to understand how receptor solubilization and subsequent affinity purification in different detergents affect nAChR–lipid interactions, we solubilized nAChR-enriched *T. californica* membranes in nine (9) different detergents and measured the amount of phospholipids associated with nAChR prior to- and following affinity purification (Table 2). We found that the initial detergent solubilization step did not significantly deplete native nAChR lipids in any of the detergents, with Anapoe-$C_{12}E_9$, BigCHAP and LDAO retaining the highest amounts of native lipids. However, native lipids were depleted in the subsequent affinity purification step, to levels at or below the previously-reported functional thresholds (Jones et al. 1988) in all detergents, with Anapoe-$C_{12}E_9$ and BigCHAP retaining the highest amounts of residual native lipids.

Table 2 Phospholipids and cholesterol associated with the nAChR prior to- and following affinity purification

Detergent	Detergent classification based on experimental results	nAChR prior to affinity purification		nAChR following affinity purification		
		Total phospholipid (nmoles)[a]	Total cholesterol (nmoles)[a]	Total phospholipid (nmoles)[a]	Total cholesterol (nmoles)[a]	Molar ratio of lipids per-mole of nAChR[a,b]
Sodium cholate	Lipid analog	1398	1036	33	14	18
CHAPS	Lipid analog	1718	334	25	20	16
FC-12	Lipid analog	1836	183	53	34	28
BigCHAP	Retained residual amounts of native lipid	2320	263	53	90	40
Anapoe-$C_{12}E_9$	Retained residual amounts of native lipid	3001	1478	166	180	53
Cymal-6	Non-lipid analog	1261	150	22	28	9
DDM	Non-lipid analog	1780	814	25	41	20
LDAO	Non-lipid analog	2557	1610	5	34	17
OG	Non-lipid analog	1875	659	6	17	9

Lipid analysis results for detergent-solubilized crude membranes and affinity-purified nAChR in each individual detergent, including detergent classification, residual native lipid amounts and molar ratio of lipids per-mole of nAChR

[a] Total phospholipid and cholesterol amounts for both sucrose gradient membranes and affinity column samples were normalized using protein concentration values in mg/mL obtained from BCA™ assay measurements

[b] Molar ratio = $\frac{\text{Total phospholipid + Total cholesterol following ligand-affinity column purification (nmoles)}}{\text{nmoles of affinity-purified nAChR}}$

Detergent Effects on nAChR Function and Stability

In order to assess how the use of different detergents during affinity purification affects nAChR function and stability, we solubilized nAChR-enriched *T. californica* membranes, affinity-purified the receptor and assayed: (1) nAChR function by measuring ion channel conductance in planar lipid bilayers and (2) nAChR stability and aggregation by A-SEC. We found that the detergents displayed similar biophysical behaviors depending on whether they were: lipid-analogue detergents, including sodium cholate, CHAPS and FC-12; detergents retaining residual amounts of native lipids, including Anapoe-$C_{12}E_9$ and BigCHAP; or non-lipid-analogue detergents, including Cymal-6, DDM, LDAO and OG (Table 1).

Planar Bilayer Experimental Controls: Establishment of Baseline Measurements and α-BTX Blocking of Ion Channel Currents

A baseline for nAChR ion channel function measurements was established with affinity-purified nAChR in each detergent prior to each experiment, showing no signal in the absence of Carb at −70 mV membrane potential (Fig. 1a shows an example for sodium cholate). Incubation of affinity-purified nAChR in sodium cholate with α-BTX receptor antagonist following affinity purification completely blocked ion channel currents in the presence of 0.5 μM Carb (Fig. 1b).

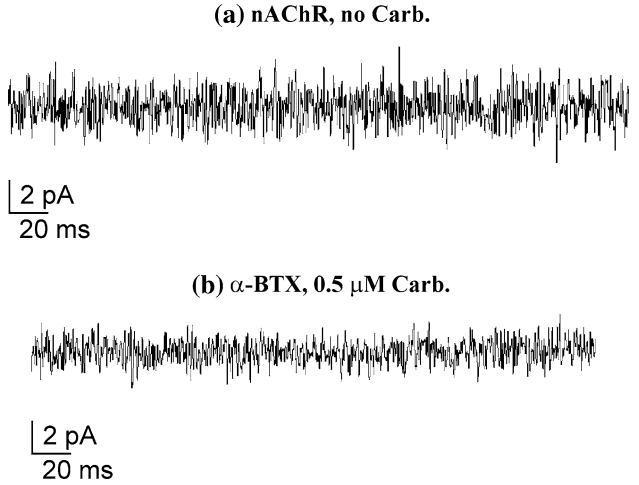


Fig. 1 Planar lipid bilayer experimental controls using affinity-purified nAChR in sodium cholate. Planar lipid bilayer ion channel function assay controls at −70 mV membrane potential: (**a**) affinity-purified nAChR with no ligand added and (**b**) nAChR incubated with α-BTX following affinity column purification in the presence of 0.5 mM Carb

Lipid-Analogue Detergents: Sodium Cholate, CHAPS and FC-12

Despite their structural differences (Table 1), these three detergents showed remarkably similar results in all categories. Figure 2a, c and e shows planar bilayer ion channel traces for sodium cholate, FC-12 and CHAPS, with clearly

defined opening–closing events; electrophysiological data summarizing nAChR functional parameters is shown in Table 3. The overall data compares favorably to previous studies of Con-A purified nAChR in sodium cholate, reconstituted into soybean lipid vesicles prior to planar lipid bilayer experiments (Nelson et al. 1980). These results suggest that despite significant lipid depletion upon affinity purification (Table 2), the receptor is fully functional in lipid-analogue detergents.

A-SEC stability data for these detergents showed some aggregation upon affinity column purification, indicating that the receptor was slightly destabilized, which is consistent with the observed lipid depletion. Detergent-dependent variations in the monomer-dimer ratios were also observed after purification when compared to detergent-solubilized, nAChR-enriched membranes. Sodium cholate showed ~13% aggregate and decreases of ~7% in both monomer and dimer (Fig. 2b). FC-12 showed the lowest aggregation of any detergent in this study at ~7%, with ~3% increase in the dimer and ~10% decrease in the monomer (Fig. 2d). CHAPS, which is structurally similar to sodium cholate (Table 1), showed a comparable amount of aggregate (~15%), with ~20% decrease in the dimer and ~6% increase in the monomer (Fig. 2f).

EM data were partially consistent with A-SEC, with clearly visible nAChR molecules in both sodium cholate and FC-12 (Fig. 3a and c), while considerable aggregation was apparent for CHAPS (Fig. 3e). SDS-PAGE gels in Fig. 3b, d and f show that the nAChR is mostly pure in all three detergents, specially sodium cholate, showing the characteristic four-band pattern with minimal contaminants.

Detergents in which the nAChR Retained Higher Amounts of Residual Native Lipid Following Affinity Purification: Anapoe-$C_{12}E_9$ and BigCHAP

Table 2 shows that Anapoe-$C_{12}E_9$ and BigCHAP retained noticeably higher residual amounts of native lipids following purification compared to other detergents. Figure 4a and c show single-channel currents for Anapoe-$C_{12}E_9$ and BigCHAP, with less well-defined ion channel opening–closing events than those for lipid-analogue detergents. Consequently, lower single-channel conductance and open channel times were observed, specially for BigCHAP, while the number of events per bilayer experiment in both detergents was similar to those observed for lipid-analogue detergents (Table 3).

A-SEC stability data shows noticeable increases in the amount of aggregation for both detergents upon affinity purification, which indicates lower nAChR stability compared to lipid-analogue detergents despite the slightly higher amounts of residual native lipid, correlating with the observed reduction in ion channel function. Similar to lipid-analogue detergents, Anapoe-$C_{12}E_9$ and BigCHAP showed a mixture of monomer–dimer species upon detergent solubilization of the native membranes that was altered upon nAChR purification. Anapoe-$C_{12}E_9$ showed ~20% increase in the aggregate, along with decreases of ~5% in the dimer and ~15% in the monomer (Fig. 4b), while BigCHAP showed the same ~20% increase in aggregate but with a decreases of ~16% in the dimer and ~4% in the monomer (Fig. 4d).

EM data for these detergents show individual nAChR pentamers visible in Anapoe-$C_{12}E_9$, similar to sodium cholate and FC-12 (Fig. 4e), while BigCHAP exhibited

Table 3 nAChR planar bilayer assay data summarizing nAChR ion channel function parameters

Detergent	Detergent classification based on experimental results	Average number of processed events per-bilayer (N)	Channel current (mean ± SD) pA	Open channel time (mean ± SD) ms
Cholate	Lipid analog	214	−(2.48 ± 0.03)	3.0 ± 0.1
CHAPS	Lipid analog	262	−(2.69 ± 0.04)	3.51 ± 0.04
FC-12	Lipid analog	285	−(2.48 ± 0.02)	2.52 ± 0.07
Anapoe-$C_{12}E_9$	Retained residual amounts of native lipid	274	−(1.77 ± 0.02)	2.40 ± 0.03
BigCHAP	Retained residual amounts of native lipid	235	−(1.76 ± 0.05)	2.05 ± 0.05
Cymal-6	Non-lipid analog	N/A	N/A	N/A
DDM	Non-lipid analog	N/A	N/A	N/A
LDAO	Non-lipid analog	N/A	N/A	N/A
OG	Non-lipid analog	N/A	N/A	N/A

Electrophysiological parameters for affinity-purified nAChR in each individual detergent, including detergent classification, average number of processed events per- bilayer experiment (N), mean channel current and mean open channel time

N/A, Carb-induced nAChR ion channel function was not observed. For functional detergents, an average of three (3) bilayer experiments with durations of ~30 min were performed. For detergents that did not display Carb-induced nAChR ion channel function, an average of six (6) bilayer experiments with durations of ~45 min were performed. Histograms from which mean ion channel current and mean open channel time electrophysiological parameter values were obtained are shown as supplementary materials

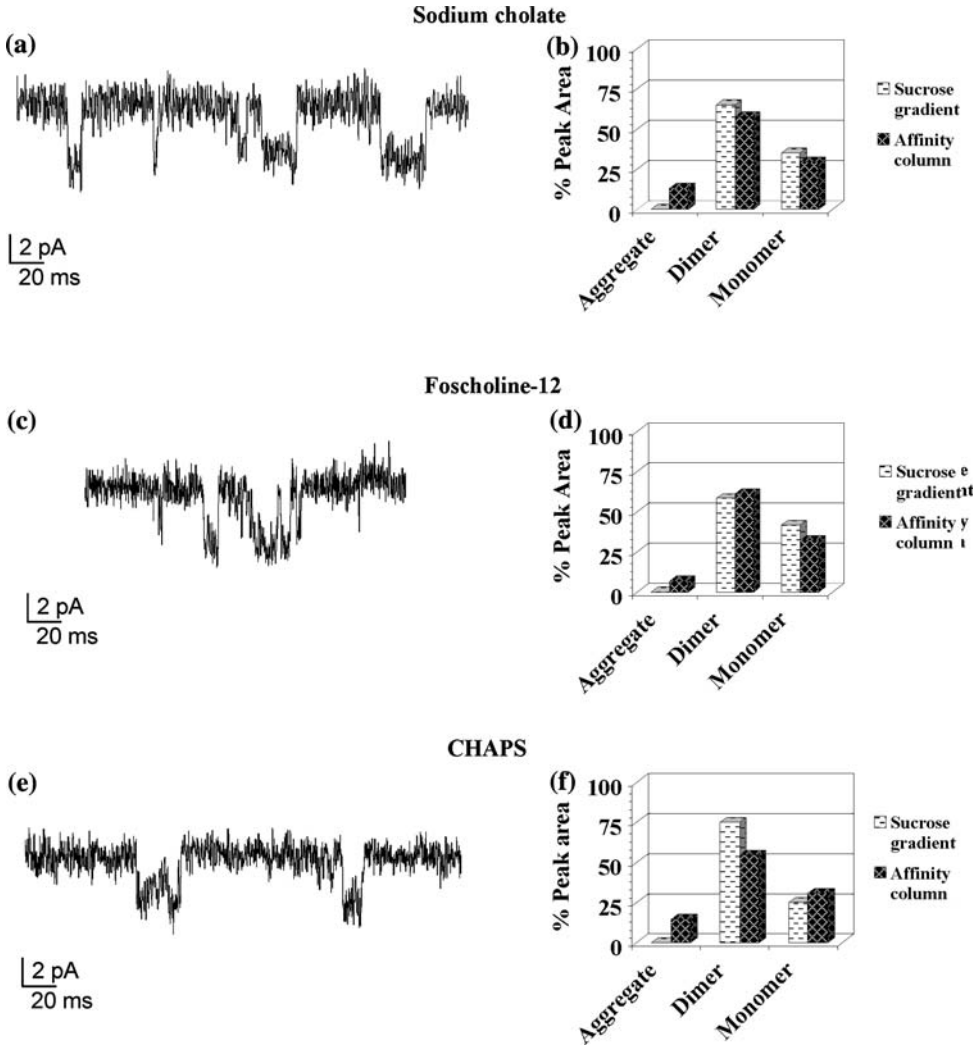


Fig. 2 Ion channel function and stability assays of affinity-purified nAChR in lipid-analog detergents. Planar bilayer current traces at −70 mV membrane potential in the presence of 0.5 μM Carb. (*left panels*) for: (**a**) Sodium cholate, (**c**) FC-12 and (**e**) CHAPS. A-SEC stability assay percent (%) peak areas (*right panels*) for sucrose gradient detergent-solubilized membranes (*white background*) and ligand affinity-purified nAChR (*black background*) in (**b**) Sodium cholate (sucrose gradient % peak areas: 65% dimer, 35% monomer; affinity column % peak areas: 13% aggregate, 58% dimer, 29% monomer), (**d**) FC-12 (sucrose gradient % peak areas: 58% dimer, 42% monomer; affinity column % peak areas: 7% aggregate, 61% dimer, 32% monomer) and (**f**) CHAPS (sucrose gradient % peak areas: 75% dimer, 25% monomer; affinity column % peak areas: 15% aggregate, 55% dimer, 31% monomer)

considerably more aggregation, similar to CHAPS (Fig. 4g). SDS-PAGE gels in Fig. 4f and h show noticeably more contaminants than what was observed for lipid-analogue detergents, specially for BigCHAP, perhaps as a result of the residual native lipids, while the characteristic nAChR bands are still observable.

Non-Lipid-Analogue Detergents: Cymal-6, DDM, LDAO and OG

Native lipid depletion in these detergents was comparable to that in lipid-analogue detergents (Table 2), but ion channel function was completely suppressed following nAChR purification (Table 3).

A-SEC stability assays for these detergents showed similar effects on monomer–dimer ratios upon detergent solubilization of the native membranes as observed for other detergents. However, following affinity column purification and native lipid depletion, significant increases in aggregation were observed, higher than for all of the other detergents in the study. Cymal-6 showed the lowest aggregation with ∼23%, similar to Anapoe-$C_{12}E_9$ and BigCHAP, with nearly identical ∼12% reductions in both dimer and monomer (Fig. 5a). DDM, which is structurally similar to Cymal-6 (Table 1), showed ∼30% aggregation, with a similar ∼10% decrease in the dimer but a nearly double decrease in the monomer of ∼20% (Fig. 5b). LDAO showed ∼33% aggregation, slightly higher than Cymal-6 and DDM, with decreases of ∼20% in the dimer and ∼13% in the monomer, almost the opposite results to DDM (Fig. 5c). OG showed nearly double the amount of aggregation of the other non-lipid-analogue detergents at ∼60%, the highest by far of the group, with ∼44% reduction of the dimer and complete depletion of the monomer (Fig. 5d).

EM data show clearly visible pentamers in both Cymal-6 and DDM (Fig. 5e and g), while significant aggregation was apparent in both LDAO and OG (Fig. 5i and k). SDS-PAGE gels in Fig. 5f, h, j and l show minimal contaminants, with the characteristic nAChR band pattern

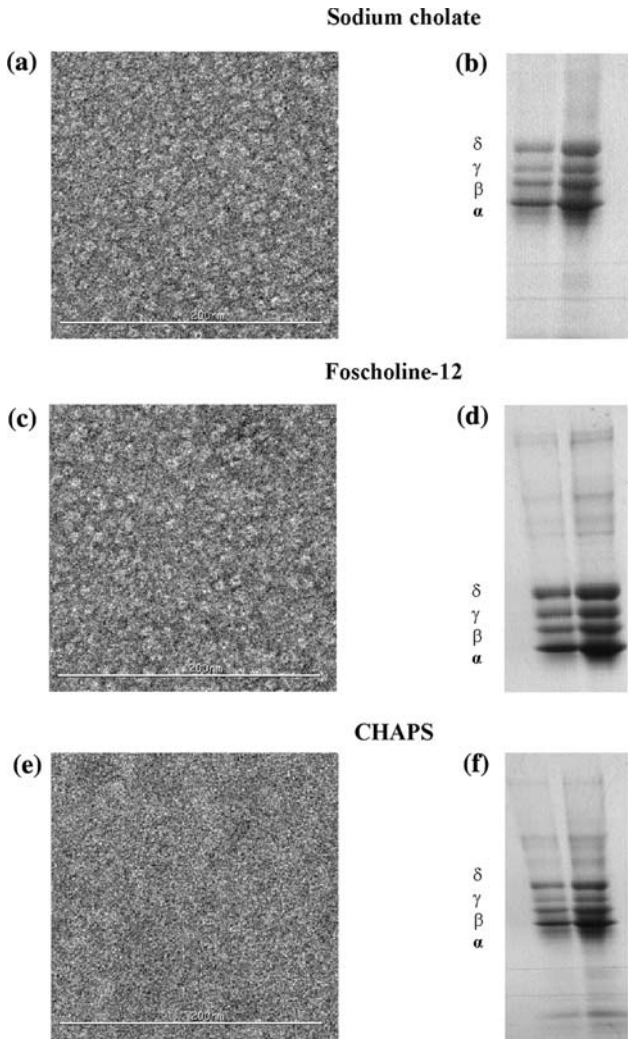


Fig. 3 EM and SDS-PAGE of affinity-purified nAChR in lipid-analog detergents. Negative-stain EM (*left panels*) for: (**a**) Sodium cholate, (**c**) FC-12 and (**e**) CHAPS; scale bar = 200 nm. SDS-PAGE gels (*right panels*) for: (**b**) Sodium cholate, (**d**) FC-12 and (**f**) CHAPS

comparable to FC-12 and CHAPS and a marked improvement over Anapoe-$C_{12}E_9$ and BigCHAP, but not as clean as that observed for sodium cholate.

Discussion

We found considerable differences in all biophysical parameters measured between detergent-solubilized sucrose gradient membranes and affinity-purified nAChR, mainly as a result of alteration of the native lipid composition. Detergent solubilization of sucrose gradient nAChR-enriched membranes did not result in significant native lipid depletion (Table 2), and nAChR stability was not substantially affected. A-SEC assays showed no aggregation upon detergent solubilization, consistent with the observed minimal lipid depletion, and both nAChR monomer and dimer species were observed in all detergents as previously reported (Anholt et al. 1980; Figs. 2, 4 and 5). Affinity column purification caused significant lipid depletion in all detergents (Table 2), to levels at or below the previously reported functional thresholds (Jones et al. 1988). Lipid depletion destabilized the receptor, as evidenced by higher aggregation levels, altered monomer–dimer ratios and effects on ion channel function, in a detergent-dependent manner. Agonist and antagonist binding was unaffected by the identity of the detergent, since we were able to purify the nAChR in all detergents by ligand-affinity, and formation of the nAChR-Alexa Fluor 488 α-BTX antagonist complex, a necessary condition for detection in A-SEC stability assays, occured in all detergents (Figs. 2, 4 and 5).

Based on the similarity of their behavior in the biophysical characterization assays, the detergents were grouped into three categories (Table 1): lipid-analogue detergents, including sodium cholate, CHAPS and FC-12; detergents retaining residual amounts of native lipids, including Anapoe-$C_{12}E_9$ and BigCHAP, and non-lipid-analogue detergents, including Cymal-6, DDM, LDAO and OG. Three experimental outcomes were observed upon nAChR purification: (1) stable and functional nAChR in lipid-analogue detergents, despite native lipid depletion, (2) moderately stable and functional nAChR in detergents that retained residual amounts of native lipids, and (3) unstable and non-functional nAChR in non-lipid-analogue detergents. We propose an equilibrium model to explain these results (Fig. 6), where k_{sg} (sucrose gradient constant) governs the monomer–dimer equilibrium for nAChR-enriched, sucrose gradient detergent-solubilized membranes. Upon affinity purification and native lipid depletion, k_a (overall affinity-purified nAChR constant) governs the monomer–dimer equilibrium, while k_{am} (affinity-purified nAChR monomer constant) and k_{ad} (affinity-purified nAChR dimer constant) govern depletion of the monomer and dimer states, respectively, to form the aggregate. The reversibility of this aggregation remains to be established, but the observed depletion of the dimer and monomer species in a detergent-dependent manner suggests that both nAChR species could aggregate, perhaps by different mechanisms. It is very likely that, to some extent, aggregation could lead to partial or completely irreversible nAChR denaturation, as evidenced by the correlation between increased aggregation and loss of ion channel function.

Native lipid depletion upon receptor purification, a necessary step for structural studies, could expose the nAChR transmembrane domains to the aqueous buffer environment, leading to premature aggregation and loss of ion channel function, as observed for some detergents.

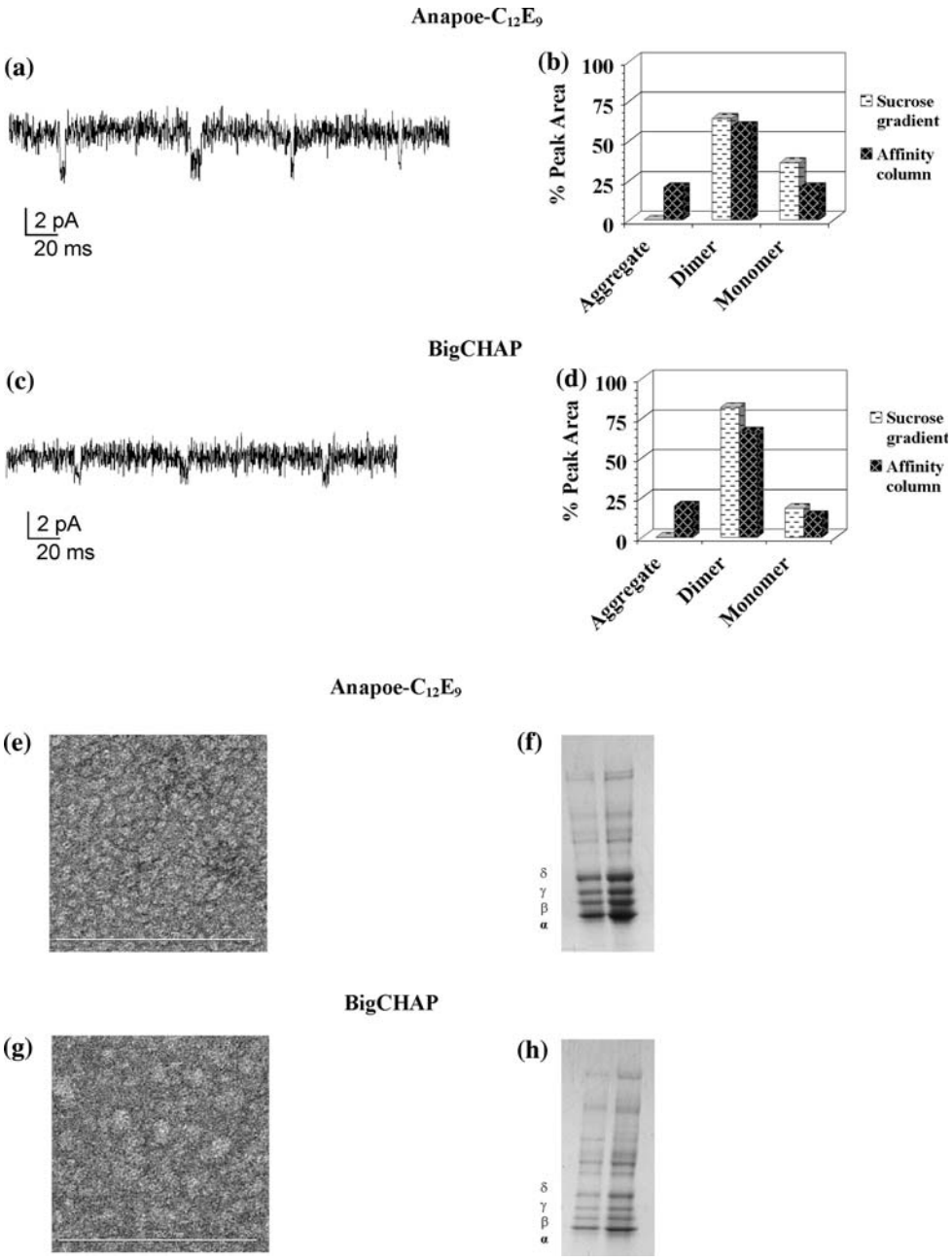


Fig. 4 Ion channel function and stability assays, EM and SDS-PAGE of affinity-purified nAChR in detergents retaining residual amounts of native lipid. Planar bilayer current traces at −70 mV membrane potential in the presence of 0.5 μM Carb. (*top left panels*) for: (**a**) Anapoe-$C_{12}E_9$ and (**c**) BigCHAP and A-SEC stability assay percent (%) peak areas (*top right panels*) for sucrose gradient detergent-solubilized membranes (*white background*) and ligand affinity-purified nAChR (*black background*) in (**b**) Anapoe-$C_{12}E_9$ (sucrose gradient % peak areas: 64% dimer, 36% monomer; affinity column % peak areas: 20% aggregate, 59% dimer, 21% monomer) and (**d**) BigCHAP (sucrose gradient % peak areas: 82% dimer, 18% monomer; affinity column % peak areas: 20% aggregate, 66% dimer, 14% monomer). Negative-stain EM for: (**e**) Anapoe-$C_{12}E_9$ and (**g**) BigCHAP (*bottom left panels*); scale bar = 200 nm. SDS-PAGE gels for: (**f**) Anapoe-$C_{12}E_9$ and (**h**) BigCHAP (*bottom right panels*)

The structural features of lipid-analogue detergents could have allowed them to interact favorably with nAChR transmembrane domains, perhaps contributing to the ~20–45 moles of lipid/per-mole of receptor functional threshold (Jones et al. 1988), thus preserving nAChR functionality in spite of native lipid depletion. The comparatively low aggregation observed in A-SEC and EM for lipid-analogue detergents suggests that k_{am} and k_{ad} were small (Fig. 6), indicating that these detergents are clearly the best choice for maintaining both stability and ion channel function upon affinity purification while achieving a reasonably high level of purity. One of the drawbacks of lipid analog detergents is that under these affinity purification conditions, a nearly total native lipid depletion was observed.

Detergents retaining slightly higher amounts of residual native lipids showed aggregation and ion channel function parameters with intermediate values between those observed for lipid- and non-lipid analogue detergents. Thus, there is a shift of k_{am} and k_{ad} toward aggregation (Fig. 6), but not enough to cause loss of ion channel function. Thus, the presence of increased amounts of residual native lipids appears to offset the apparent structural effect patterns observed for lipid-and non-lipid analog detergents in the absence of these lipids. BigCHAP required two (2) to four (4) times more detergent for

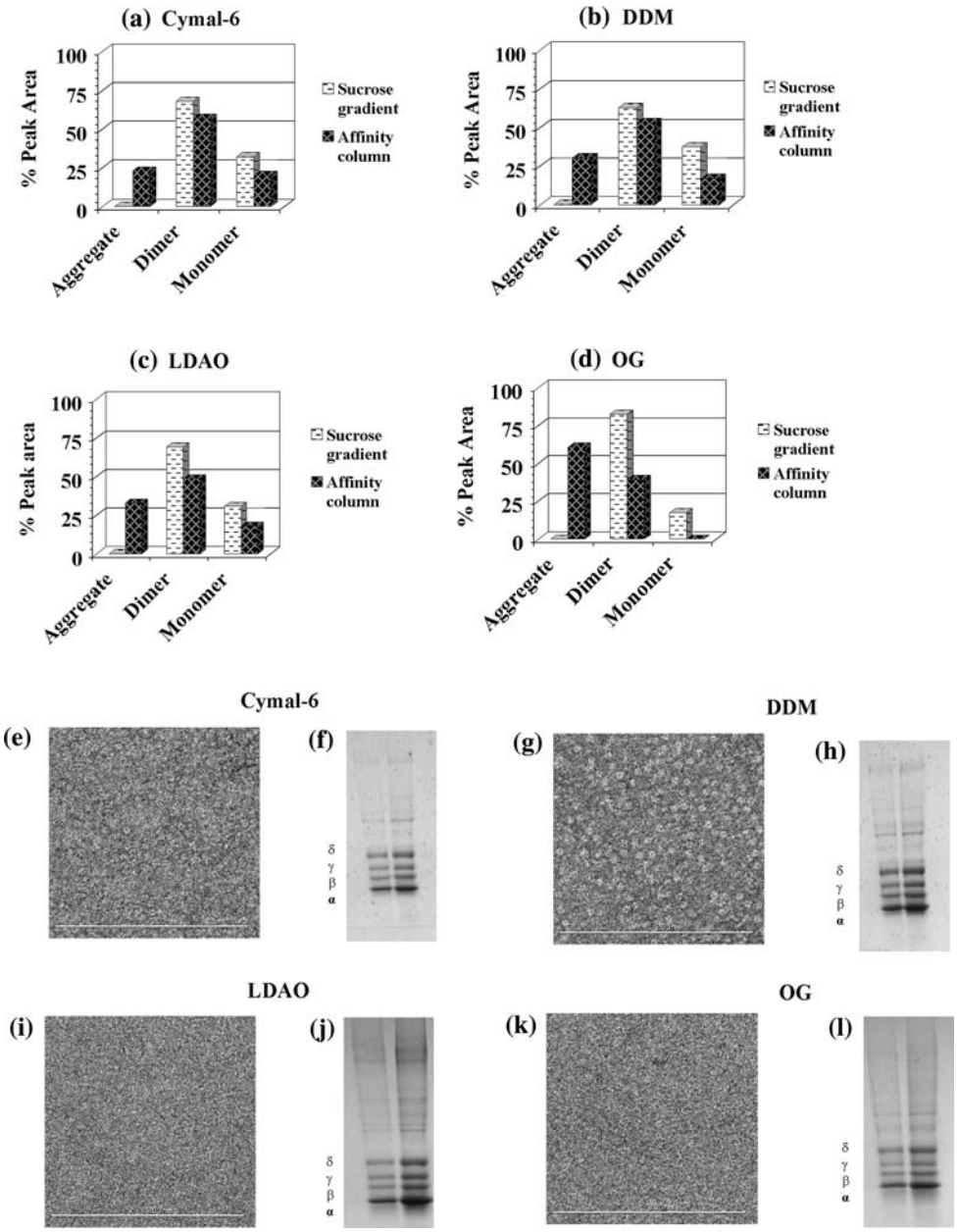


Fig. 5 Stability assays, EM and SDS-PAGE of affinity-purified nAChR in non-lipid analog detergents. A-SEC stability assay percent (%) peak areas (*top of the figure*) for sucrose gradient detergent-solubilized membranes (*white background*) and ligand affinity-purified nAChR (*black background*) in: (**a**) Cymal-6 (sucrose gradient areas % peak areas: 68% dimer, 32% monomer; affinity column % peak areas: 23% aggregate, 57% dimer, 20% monomer); (**b**) DDM (sucrose gradient % peak areas: 63% dimer, 37% monomer; affinity column % peak areas: 30% aggregate, 53% dimer, 17% monomer); (**c**) LDAO (sucrose gradient % peak areas: 69% dimer, 31% monomer; affinity column % peak areas: 33% aggregate, 49% dimer, 18% monomer) and (**d**) OG (sucrose gradient % peak areas: 83% dimer, 17% monomer; affinity column % peak areas: 61% aggregate, 39% dimer, 0% monomer). Negative-stain EM (*bottom of the figure*) for: (**e**) Cymal-6, (**g**) DDM, (**i**) LDAO and (**k**) OG; scale bar = 200 nm. SDS-PAGE gels for: (**f**) Cymal-6, (**h**) DDM, (**j**) LDAO and (**l**) OG

nAChR solubilization than the other detergents, which could cause partial denaturation. Furthermore, its comparatively-bulky functional group as opposed to the smaller functional groups in CHAPS and sodium cholate (Table 1) could have prevented favorable interactions with the nAChR transmembrane domains. The presence of residual native lipids in Anapoe-$C_{12}E_9$ appeared to allow this non-lipid-analogue detergent to partially compensate for the potential adverse effects of its non-lipid-analogue structure on nAChR stability and ion channel function. Previous planar bilayer studies in Triton X-100-solubilized, Tween-80-purified nAChR, both non-lipid analog detergents, prior to reconstitution into 1,3-SMPC (1-stearoyl-3-myristoyl-glycero-2-phosphocholine) have shown ion channel function (Boheim et al. 1981). Some of the ion channel opening-closing events on this study were as well-defined as those for sodium cholate (Nelson et al. 1980) while others were not, which would be consistent with our own results for Anapoe-$C_{12}E_9$ and BigCHAP. The fact that both of these detergents retain residual amounts of native lipid following nAChR affinity purification, as opposed to the nearly-total native lipid depletion observed for all other detergents, makes them viable choices for nAChR purification. However, the observed reduction in ion channel function when compared to lipid-analog detergents, despite the presence of residual native lipids, could point to potentially detrimental detergent–lipid–protein interactions.

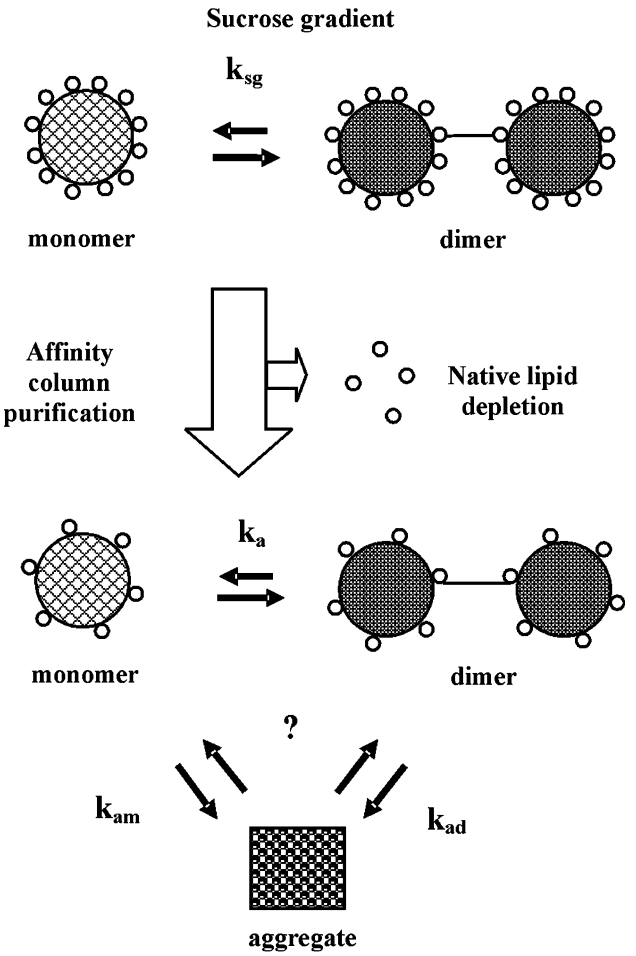


Fig. 6 Proposed nAChR monomer-dimer equilibrium scheme. The figure describes a possible mechanism to explain the observed experimental results based on how the nAChR monomer-dimer equilibrium could be affected during affinity purification as a result of native lipid depletion

Non-lipid-analogue detergents significantly increased aggregation and native lipid depletion upon affinity purification, as observed in both A-SEC (specially in LDAO and OG) and EM (except Cymal-6 and DDM), along with a total loss of ion channel function. These results are consistent with the apparent inability of these detergents to effectively interact with residual lipids and shield potentially exposed nAChR transmembrane domains from the aqueous buffer environment. These potential structural constraints would increase k_{am} and k_{ad} values (Fig. 6), shifting the equilibrium towards aggregation.

Previous studies with OG-solubilized crude membranes reconstituted in soybean lipid vesicles showed no nAChR ion channel flux (Anholt et al. 1981). Direct functional measurements of detergent-solubilized membranes were attempted but were ultimately unsuccessful in our study, due to the significant amount of non-nAChR proteins incorporated into the bilayer, which almost always led to premature membrane breakage. However, our overall results suggest that since detergent-solubilized membranes did not show significant native lipid depletion or decreased stability in A-SEC, the nAChR should be stable and functional under these conditions. It is possible that non-lipid analog detergents could interact with other critical domains to suppress ion channel function, which would be consistent with a direct negative effect of OG on the nAChR, as reported by Anholt et al. 1981.

Our results showed that upon nAChR affinity purification, native lipid depletion caused destabilization and loss of ion channel function. This finding is in disagreement with previously-published results suggesting that OG-solubilized, affinity-purified nAChR showed ion channel flux, although for these particular studies, the affinity-purified nAChR was reconstituted in native lipid vesicles (Paraschos et al. 1982). It is possible that the observed ion channel flux was produced by a small fraction of intact nAChR channels that were protected from detergent exposure by either: (1) the reduced amount of detergent used during solubilization, which at ~34 mM was barely above the OG CMC value of ~23.4 mM, (2) the presence of native lipids in reconstituted vesicles, or both. This discrepancy highlights the potential advantages of the methodologies presented in this study, which more closely resemble the actual detergent-solubilized environment in which most structural studies would be performed. In our study, the protein remained in contact with the detergent at all times, as opposed to the complete detergent removal necessary for reconstitution in lipid vesicles, which could reverse the potentially-detrimental effects of OG or other detergents.

It is clear that non-lipid-analogue detergents, at least the ones tested in this study, are not suitable for nAChR affinity purification in the absence of native or exogenous lipids, because of their negative effects on nAChR ion channel function and stability. Certainly, the results observed by Paraschos et al. 1982 for OG, Boheim et al. (1981) for Triton-X100/Tween-80, and our own results for Anapoe-$C_{12}E_9$ and BigCHAP suggest that the presence of exogenous or residual native lipids could preserve or even restore ion channel function in non-lipid-analogue detergents. Perhaps addition of native or exogenous lipids during purification to non-lipid-analogue detergents could increase nAChR stability to the levels required to restore ion channel function, something that could be addressed in a future study.

The overall results of this study provide suitable biophysical methodologies to probe detergent–lipid–protein interactions prior to membrane protein structural studies, while perhaps shedding light on some of the problems that have hampered these studies over the past few decades. The fact that all of these techniques can be used on the

detergent-solubilized state, which more closely mimics the actual environment to which the protein is exposed to prior to structural studies, provides a clear advantage over previous studies performed in native membranes or reconstituted lipid vesicles. The combination of A-SEC and EM as sample stability and aggregation state prediction tools, as well as planar lipid bilayers with the PMAL protein delivery method to probe nAChR ion channel function, and the more conventional GC-FID and GC-MSD for lipid analyses, has provided valuable insight into the overall state of the protein sample in the detergent-solubilized state which was not previously available.

In conclusion, the nAChR purified under these conditions is by no means in its optimal state for structural studies, and significant challenges remain in terms of obtaining a homogenous, stable and functional sample. These problems would need to be addressed in future biophysical or structural studies, and this study provides an important starting point in that direction. Further optimization of nAChR purification conditions using biophysical techniques as probes, perhaps through the addition of native or exogenous lipids during or after affinity purification, could lead to significant improvements in nAChR stability in the detergent-solubilized state, increasing the likelihood of success in future nAChR and membrane protein structural studies.

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References

Anholt R, Lindstrom J, Montal M (1980) Functional equivalence of monomeric and dimeric forms of purified acetylcholine receptors from *Torpedo californica* in reconstituted lipid vesicles. Eur J Biochem 109:481–487

Anholt R, Lindstrom J, Montal M (1981) Stabilization of acetylcholine receptor channels by lipids in cholate solution and during reconstitution in vesicles. J Biol Chem 256:4377–4387

Bhushan A, McNamee MG (1990) Differential scanning calorimetry and Fourier transform infrared analysis of lipid–protein interactions involving the nicotinic acetylcholine receptor. Biochim Biophys Acta 1027:93–101

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Boheim G, Hanke W, Barrantes FJ, Eibl H, Sakmann B, Fels G, Maelicke A (1981) Agonist-activated ionic channels in acetylcholine receptor reconstituted into planar lipid bilayers. Proc Natl Acad Sci USA 78:3586–3590

Cheng A, Leung A, Fellmann D, Quispe J, Suloway C, Pulokas J, Abeyrathne PD, Lam JS, Carragher B, Potter CS (2007) Towards automated screening of two-dimensional crystals. J Struct Biol 160:324–331

da Costa CJ, Ogrel AA, McCardy EA, Blanton MP, Baenziger JE (2002) Lipid–protein interactions at the nicotinic acetylcholine receptor. A functional coupling between nicotinic receptors and phosphatidic acid-containing lipid bilayers. J Biol Chem 277:201–208

Dellisanti CD, Yao Y, Stroud JC, Wang ZZ, Chen L (2007) Crystal structure of the extracellular domain of nAChR alpha1 bound to alpha-bungarotoxin at 1.94 Å resolution. Nat Neurosci 10:953–962; erratum in Nat Neurosci (2007) 10:1222

Fong TM, McNamee MG (1986) Correlation between acetylcholine receptor function and structural properties of membranes. Biochemistry 25:830–840

Hamouda AK, Sanghvi M, Sauls D, Machu TK, Blanton MP (2006) Assessing the lipid requirements of the *Torpedo californica* nicotinic acetylcholine receptor. Biochemistry 45:4327–4337

Hertling-Jaweed S, Bandini G, Müller-Fahrnow A, Dommes V, Hucho F (1988) Rapid preparation of the nicotinic acetylcholine receptor for crystallization in detergent solution. FEBS Lett 241:29–32

Hilf RJ, Dutzler R (2008) X-ray structure of a prokaryotic pentameric ligand-gated ion channel. Nature 452:375–379

Jones OT, Eubanks JH, Earnest JP, McNamee MG (1988) A minimum number of lipids are required to support the functional properties of the nicotinic acetylcholine receptor. Biochemistry 27:3733–3742

Karlin A (2002) Emerging structure of the nicotinic acetylcholine receptors. Nat Rev Neurosci 3:102–114

Karlin A, Akabas MH (1995) Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins. Neuron 15:1231–1244

Martínez KL, Gohon Y, Corringer PJ, Tribet C, Mérola F, Changeaux JP, Popot JL (2002) Allosteric transitions of *Torpedo* acetylcholine receptor in lipids, detergent and amphipols: molecular interactions vs. physical constraints. FEBS Lett 528:251–256

Miyazawa A, Fujiyoshi Y, Unwin N (2003) Structure and gating mechanism of the acetylcholine receptor pore. Nature 423:949–955

Nelson N, Anholt R, Lindstrom J, Montal M (1980) Reconstitution of purified acetylcholine receptors with functional ion channels in planar lipid bilayers. Proc Natl Acad Sci USA 77:3057–3061

Ochoa EL, Dalziel AW, McNamee MG (1983) Reconstitution of acetylcholine receptor function in lipid vesicles of defined composition. Biochim Biophys Acta 727:151–162

Paraschos A, González-Ros JM, Martínez-Carrión M (1982) Acetylcholine receptor from *Torpedo*. Preferential solubilization and efficient reintegration into lipid vesicles. Biochim Biophys Acta 691:249–260

Potter CS, Pulokas J, Smith P, Suloway C, Carragher B (2004) Robotic grid loading system for a transmission electron microscope. J Struct Biol 146:431–440

Schindler H, Quast U (1980) Functional acetylcholine receptor from *Torpedo marmorata* in planar membranes. Proc Natl Acad Sci USA 77:3052–3056

Schürholz T, Kehne J, Gieselmann A, Neumann E (1992) Functional reconstitution of the nicotinic acetylcholine receptor by CHAPS dialysis depends on the concentrations of salt, lipid, and protein. Biochemistry 31:5067–5077

Suloway C, Pulokas J, Fellmann D, Cheng A, Guerra F, Quispe J, Stagg S, Potter CS, Carragher B (2005) Automated molecular microscopy: the new Leginon system. J Struct Biol 151:41–60

Unwin N (2005) Refined structure of the nicotinic acetylcholine receptor at 4 Å resolution. J Mol Biol 346:967–989

Walker JW, Takeyasu K, McNamee MG (1982) Activation and inactivation kinetics of *Torpedo californica* acetylcholine receptor in reconstituted membranes. Biochemistry 21:5384–5389