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Assessment of the functionality and stability of detergent purified nAChR from *Torpedo* using lipidic matrixes and macroscopic electrophysiology

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ABSTRACT

In our previous study we examined the functionality and stability of nicotinic acetylcholine receptor (nAChR)-detergent complexes (nAChR-DCs) from affinity-purified *Torpedo californica* (Tc) using fluorescence recovery after photobleaching (FRAP) in Lipidic Cubic Phase (LCP) and planar lipid bilayer (PLB) recordings for phospholipid and cholesterol like detergents. In the present study we enhanced the functional characterization of nAChR-DCs by recording macroscopic ion channel currents in *Xenopus* oocytes using the two electrode voltage clamp (TEVC). The use of TEVC allows for the recording of macroscopic currents elicited by agonist activation of nAChR-DCs that assemble in the oocyte plasma membrane. Furthermore, we examined the stability of nAChR-DCs, which is obligatory for the nAChR crystallization, using a 30 day FRAP assay in LCP for each detergent. The present results indicate a marked difference in the fractional fluorescence recovery (ΔFFR) within the same detergent family during the 30 day period assayed. Within the cholesterol analog family, sodium cholate and CHAPSO displayed a minimum ΔFFR and a mobile fraction (MF) over 80%. In contrast, CHAPS and BigCHAP showed a marked decay in both the mobile fraction and diffusion coefficient. nAChR-DCs containing phospholipid analog detergents with an alkylphosphocholine (FC) and lysofoscholine (LFC) of 16 carbon chains (FC-16, LFC-16) were more effective in maintaining a mobile fraction of over 80% compared to their counterparts with shorter acyl chain (C12, C14). The significant differences in macroscopic current amplitudes, activation and desensitization rates among the different nAChR-DCs evaluated in the present study allow to dissect which detergent preserves both, agonist activation and ion channel function. Functionality assays using TEVC demonstrated that LFC16, LFC14, and cholate were the most effective detergents in preserving macroscopic ion channel function, however, the nAChR-cholate complex display a significant delay in the ACh-induce channel activation. In summary, these results suggest that the physical properties of the lipid analog detergents (headgroup and acyl chain length) are the most effective in maintaining both the stability and functionality of the nAChR in the detergent solubilized complex.

1. Introduction

Integral membrane proteins (IMP), such as the nicotinic acetylcholine receptor (nAChR) from the ligand gated ion channels (LGIC), are known to mediate cell communication, regulation, and their role is essential for normal physiological functions. The nAChR from *Torpedo californica* (*Tc*) is a hetero-pentameric ion channel with a pseudosymetrical arrangement around a cation selective pore. The nAChRs have been extensively studied because of their implication in several disorders, such as Congenital Myasthenic Syndromes (CMS) [1–4], Alzheimer's disease (AD) [5], Parkinson's (PD) disease [6], and other brain pathologies [7].

Abbreviations: nAChR-DCs, nicotinic acetylcholine receptors detergent complexes; *Tc*, *Torpedo californica*; *Tm*, *Torpedo marmorata*; PLB, planar lipid bilayer; TEVC, two electrode voltage clamp; LCP, Lipidic Cubic Phase; FC, foscholine family 12–16 carbon chain; LFC, lysofoscholine family 12–16 carbon chain; MF, mobile fraction; AChBP, acetylcholine binding protein; α-BTx, alpha bungarotoxin; F(t), fractional fluorescence recovery curves; FRAP, fluorescence recovery after PHOTOBLEACHING; ΔFFR, fractional fluorescence recovery.

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Most of the nAChR structural and functional data available today has been obtained from detergent-solubilized receptors from organs of electric rays, such as *T. Californica (Tc)*, *Torpedo nobiliana* (*Tn*) and *Torpedo marmorata* (*Tm*) [8]. Recently, studies of nAChR structural composition have been done using expression systems such as bacteria and yeast, which are not ideal because they lack the proper machinery for posttranslational modifications, such as glycosylations, and often require the production of modified versions of the receptors which in most cases involve expressing either truncated or mutated receptors, or constructing chimeras that increase stability with unknown effects on the protein structure [9]. Studies performed by Corringer [8] solved a high-resolution crystal structure of the homologous acetylcholine binding protein (AChBP) expressed in bacteria. AChBP has ~24% sequence identity with the nAChR and the structure determined to 3.3 Å resolution demonstrated similar pentameric symmetry. In 2007, a high-resolution X-ray structure of the mouse-α1 nAChR extracellular domain, which was expressed in yeast, was solved to 1.94 Å resolution [10]. In spite of all these structural studies, there is a lot of structural information that remains unknown about the transmembrane domains. Previous efforts used detergents to obtain a nAChR structure [11], however, these studies failed to produce a high resolution structure. EM studies using nAChR-enriched membranes from the electric organ of *Tm* led to a complete three-dimensional structure at 4.0 Å resolution [12,13], which showed limited structural insights as a result of the low atomic resolution. After several decades and a great deal of effort from independent laboratories the best approximation to a high-resolution structure of the nAChR has been a collection of fragmented structures from different species. A fundamental obstacle towards the achieving of high resolution structure of the nAChR is the preparation of milligram amounts of stable, homogeneous and functional nAChR-detergent complexes (nAChR-DCs). A key aspect in the preparation of suitable nAChR-DCs for structural studies is the preservation of the stability and functionality of the ion channel machinery.

There have been several unsuccessful efforts to obtain a nAChR high-resolution structure during the past three decades. Several research groups have reported crystallization conditions that yielded small protein crystals (~15 μm); however, no high-resolution X-ray diffractions for the nAChR have been reported [11,14]. Neither these nor any other groups ever succeeded in obtaining a high resolution structure of the nAChR. Over the course of the past two decades, no attempts have been made to bring back the possibility of making high quality nAChR crystals from natural source. It is important to note that the aforementioned nAChR crystallization studies [11,14] used Octyl glucoside (OG) as the principal detergent. In a previous study, we demonstrated that OG decreased the stability and abolished the functional response of the nAChR [15]. Along that line, the nAChR purified in those studies was by no means in its optimal state for structural studies. It is important to note that in recent years there have not been any reports of further efforts to obtain high quality crystals of functional and intact receptor.

In the present study we examined the macroscopic ion channel behavior of the nAChR-DCs in *Xenopus* oocytes and their stability using LCP-FRAP. Previously, we found that during solubilization, a detergent may selectively remove a native lipid specie(s) present in the native cell membrane that could be essential for protein function and/or stability [15,16]. In the present study we demonstrated that the LCP in combination with macroscopic functional assays in *Xenopus* oocytes can lead to high quality nAChR-DCs. For the first time we recorded macroscopic currents, as an indicator of the functional integrity of the detergent solubilized nAChR, prior to insertion into LCP and also evaluate the stability of detergent solubilized nAChR in LCP during a 30 day period. The stability of the nAChR-DCs was evaluated using LCP-FRAP approaches to estimate nAChR mobile fraction and diffusion coefficient that had been correlated with receptor stability and/or aggregation. Overall, the present study demonstrates that lipid-analog detergents that resemble native environment of nAChR induce a functional stabilization of the nAChR-DCs. Using these approaches we have prepared highly functional and stable nAChR-DCs that could be the excellent candidates for future structural studies of the nAChR and other related proteins.

2. Materials and methods

2.1. Crude membrane isolation, detergent solubilization and receptor purification

Crude membrane isolation, detergent solubilization, and receptor purification were carried out as described in Asmar-Rovira et al. (2008) [15]. This study examined the ion channel function and LCP mobility of the affinity-purified *T. californica* nAChR for each of the following detergents: n-dodecylphosphocholine (FC-12), n-tetradecylphosphocholine (FC-14), n-hexadecylphosphocholine (FC-16) and 1-dodecanoyl-*sn*-glycero-3-phosphocholine (LFC-12) 1-tetradecanoyl-*sn*-glycero-3-phosphocholine (LFC-14), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (LFC-16), from the phospholipid-analog or FC family, as well as CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), (*N*,*N*′-bis-[3-D-gluconamidopropyl] cholamide) (BigCHAP) and sodium cholate from the cholesterol-analog family. All detergents were obtained from Anatrace (Maumee, OH).

2.2. Injection of oocytes with crude or nAChR detergent complex

This protocol is a modified version of the protocol used by the Miledi and Morales group [17–19]. Briefly, oocytes were obtained from *Xenopus leavis* in the V or VI developmental stage and microinjected with 50 nL of 6 mg/mL of crude membrane or 3 mg/mL of 1.5 fold critical micellar concentration nAChR detergent complex, affinity purified from *Tc*. Subsequently, the oocytes were incubated at 18 °C for 16–36 h in ND-96 solution containing in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 2.5 Na-pyruvate supplemented with gentamicin (50 mg/mL), tetracycline (50 mg/mL) and theophylline (0.5 mM); and adjusted to a pH of 7.6 with NaOH.

2.3. Two electrode voltage clamp experiments (TEVC) on oocytes

For the TEVC experiments we used a modified version of previous protocols published by our group [20–22]. Briefly, membrane current recordings were performed at room temperature (21–25 °C) 16–36 h after injection, longer incubation times were tested, but oocyte viability was greatly reduced for some detergents, therefore we choose a timeframe that was favorable for all the detergents present in this study. Oocytes were placed in a 200 μL chamber that was continuously perfused with 5 mL/min of a calcium-depleted OR-2 (containing in mM: 82 NaCl, 2.5 KCl, 1 $MgCl_2$, 5 HEPES; and adjusted to a pH of 7.6 with NaOH) to elude the activation of an endogenous Ca^{2+} dependent chloride current. The macroscopic currents were induced by a 5 s application of a non-saturating concentration of acetylcholine (100 μM, to make sure that we are not underestimating the amplitudes because of desensitized receptors) through a computer control 8 channel perfusion system (VC-8, Warner Instruments, Hamden, CT) connected to a 8–1 perfusion mini manifold, at a holding potential of −70 mV using a Gene Clamp 500B amplifier (Axon Instruments, Foster City, CA). The electrodes were filled with a solution of 3 M KCl and the resistances were calculated to average 1.3 mΩ. Macroscopic currents were filtered at 100 Hz and digitized at 1000 Hz using a Digidata 1440A interface (Axon Instruments, Foster City, CA) and acquired using the Clampex 10.2, (pCLAMP 10.2 software, Molecular Devices) running on a Microsoft Windows-based computer.