Regulation of acetylcholine receptor function by cholesterol

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Abstract

The effect of cholesterol on the nicotinic acetylcholine receptor (nAChR) function has been extensively studied by several laboratories. The goal has been to characterize the dynamic lipid-protein interactions of functional nAChR in both native and reconstituted membranes. Two main hypotheses have been proposed to explain the functional sensitivity of the nAChR to cholesterol: bulk membrane fluidity and specific binding sites for cholesterol at the lipid-protein surface. However, the molecular mechanisms by which cholesterol influences nAChR function remain poorly understood. Most of the studies carried out so far have employed the Torpedo nAChR in reconstituted membranes. Few studies have examined the effect of cholesterol on nAChR function on intact cell membranes. A new approach to study cholesterol-nAChR interactions is to examine the effect of

Dedicated to the memory of Professor José del Castillo (12/25/1920-8/22/02)
An outstanding experimentalist, author, mentor and human being. His students, colleagues and friends worldwide will sorely miss him.

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cholesterol enrichment and depletion on cells expressing recombinant nACHRs. This approach, combined with electrophysiological recordings allows studying the interaction of cholesterol with lipid-exposed positions of the nACHR. The emphasis of this review will be on experiments from our laboratory, but comparisons with results from studies carried out by others using reconstituted membranes will be made whenever relevant. These new approaches have opened a new dimension to the studies of lipid effects on ion-channel function and to the structural and functional analysis of lipid-protein interactions.

Introduction

The nicotinic acetylcholine receptor (nACHR) is a member of ligand-gated ion channel family that includes the glycine receptor, the GABA-A receptor, the 5-HT3 receptor, and the neuronal nACHR. This membrane receptor is an essential component in cholinergic synaptic transmission. In the vertebrate neuromuscular junction, the binding of two agonist molecules to the receptor induces a conformational change, which opens a cation-selective channel. The flow of cations initiates membrane depolarization and causes the generation of an action potential. The nACHR is one of the few integral membrane proteins that can be studied at all levels of organization, ranging from electrophysiological recordings at intact synapses to structure-function studies in different expression systems such as human cell lines and oocytes. The relative ease with which nACHR can be isolated and purified from the Torpedo electric organ, make the Torpedo nACHR an excellent model for the study of lipid-protein interactions. Most of the information available on the sensitivity of the nACHR to the lipid environment was obtained using reconstituted membranes of purified Torpedo nACHR. Despite a wealth of experimental data from reconstituted systems, the role of membrane cholesterol in regulating nACHR function has not been completely elucidated. Recent experiments designed to analyze the effect of membrane cholesterol on the functional state of the Torpedo nACHR wild type and the αC418W, a novel lipid exposed mutation, will be described in this chapter. To provide an appropriate frame of reference for these experiments, a brief summary of the current knowledge of the nACHR structure with particular emphasis on the lipid exposed domains is provided, along with an overview of the techniques used to measure cholesterol incorporation in the oocyte membrane.

Nicotinic acetylcholine receptor structure

The nACHR from muscle and electric ray organ is an integral membrane protein comprised of four homologous polypeptide subunits in the stoichiometry of α2βγδ (for review see: 1-6). A consensus model for the nACHR topology deduced from hydrophobicity profiles for protein sequences based on cDNA sequences indicates that each subunit contains at least four membrane spanning regions (MSRs) of 20-30 amino acids denoted M1 through M4 with both N- and C-terminals located on the extracellular side (7) (see Figure 1). The primary structure of the MSRs is likely to play a crucial role in the gating and permeation properties of the channel. In the mid 1980s, the Xenopus laevis oocyte expression system combined with mutagenesis and patch-clamp techniques created a new dimension to the study of ion channels. Most of the studies that combined pharmacological, biochemical, site-directed mutagenesis and electro-
physiological data on MSRs have focused on M2 (8-18). These studies provide strong evidence supporting that M2 segments from each subunit associate about a central axis to form at least part of the ion channel pore and suggest that M2 regions are α-helical.

The overall spatial organization of the nAChR has been deduced from electron cryomicroscopy of *Torpedo* electric organ (6), but the detailed secondary and tertiary structures, in particular the M3 and M4 transmembrane domains, remain to be established. Previous topological models suggested that the four transmembrane domains are α-helical structures (19; 20). Molecular modeling studies suggest that M1 to M4 of each subunit assembles in a complex of α-helix and β-sheet structures (21). A cryo-electron microscopy of tubular arrays of *Torpedo* receptors produced a 9-Å resolution map that showed five tilted rods around a central axis of the membrane spanning domains; these were suggested to be the M2 segments and proposed to be the only α-helical structure of all transmembrane domains (22). A lack of similar density profiles at the nAChR periphery was interpreted as the possibility of a β structure for M1, M3 and M4 domains (22). Deuterium exchange studies using Fourier-Transformed Infrared Spectroscopy (FTIR) suggested that all transmembrane segments of the *Torpedo* nAChR are α-helical (23-25). A three dimensional structure of a synthetic peptide corresponding to the putative transmembrane segment M3 of the *Torpedo* a subunit also suggests an α-helical structure (26). We have found that the functional assembly of the muscle-type nAChR is more vulnerable to disruptions of α-helical secondary elements than to β-structure elements in the M4 transmembrane segment (27). To summarize, although Unwin and coworkers proposed a β-structure for the M3 and M4 transmembrane segments, such conclusion has been challenged by other studies that include photoaffinity labeling, Fourier transform infrared studies, NMR studies and mutagenesis studies (28-30).

**Structure of the lipid-exposed domains of the nAChR’s**

Among the four postulated transmembrane domains of the *Torpedo californica* nAChR, M3 and M4 are the most hydrophobic and have the lowest level of side chain
conservation. Elegant photolabeling studies by Cohen's group in Harvard have provided key structural information on the domains of the *Torpedo* nAChR that are exposed to the lipid environment. For example, the pattern of 3-trifluoromethyl-3-m-[^125]iodophenyl)-diazirine ([^125]I TID) labeling suggested that the M4 transmembrane domain of the α-subunit is α-helical based on evidence that demonstrates that residues Cys412, Met415, Cys418, Thr422, and Val425 were labeled in an α-helix pattern (31-32). A summary of the ([^125]I TID) labeling studies by Blanton and Cohen (31-32) is illustrated in Figure 2. As shown in Figure 2, there are 18 residues on the M3 domain and 12 residues in the M4 domain that are ([^125]I TID) labeled. Interestingly, the pattern of labeling suggests that the α-M4, γ-M3 and δ-M3 have the largest exposure to the lipid environment. Based on ([^125]I TID) labeling, the degree of exposure to the lipid environment is about 7 residues per subunit. In the β subunit the pattern of labeling is evenly distributed between M3 and M4, however, in the α-subunit this pattern predominates in M4, while in the γ and δ-subunits M3 labeling predominates. These remarkable studies by Cohen's group have provided key information to analyze lipid-exposed domains of the nAChR using recombinant expression of ion channels.

![Figure 2. α-Helical wheels of the M3 (TM3) and M4 (TM4) domains of the *Torpedo* nAChR. Helical view of the αβγα and δ subunits using clockwise rotation. The diagram was constructed using 3.6 amino acids per helical turn. Lipid-exposed residues for the four subunits are shown in bold, based on the[^125]-TID labeling published by Blanton and Cohen, (31-32) and Blanton, M.P. personal communication. Two proposed lipid-exposed residues the γC451 and δS437 are not shown in the diagram. The asterisk in the αC412 residue stands for it's labeling as a cholesterol-binding residue (59).](image)

**Lipid-exposed domains and their possible role on nAChR ion-channel function**

The first studies involving mutational analysis of *Torpedo* M4 residues were reported by McNamee's group on the γC451 and αC418 positions (33-34). Further
analysis of the αCys418 demonstrated that tryptophan substitution at this position dramatically prolonged channel open time at low acetylcholine concentrations (35). It was also demonstrated that γLeu440 and γMet442 located in the M4 domain of the muscle-type γ subunit, contribute to the increased channel open time characteristic of the embryonic receptor (36). Other studies in which a series of amino acid substitutions were performed at αG421 showed that only tryptophan substitutions in this M4 position were able to produce a substantial increase (10-fold) in open channel probability (37). A phenylalanine substitution at positions αC418 and αG421 produced a small increase (2-fold) in the open time constant when compared to a tryptophan substitution. Polar side chains like serine and tyrosine substitution at these positions did not produce any significant increase in the open channel constant. These results suggested that polar side chains could not establish appropriate interactions, which leads to the hypothesis that hydrophobicity of the side chain is essential to establish the interactions that produce a more stable open channel conformation. The stabilization of the open channel state estimated from the transition state energy for the αC418W mutation is about -0.8 kcal/mol per tryptophan substitution (37). Also, the stabilization effect was shown to be additive when multiple tryptophan substitutions where combined at equivalent position of the alpha and beta M4 transmembrane segment, as in the αC418WβC447W mutation. These data suggested that Van der Waals and perhaps dipole interactions at the periphery of the nAChR with the lipid interface could play a significant role in the overall mechanism of channel gating. The combined results on the M4 tryptophan substitutions raised the following question: are these effects related to disruption of helix-helix contacts, subunits contacts, allosteric interactions, or a unique interaction of the tryptophan side chain at the lipid interface of the receptor?

One hypothesis proposed to explain these results is that the position of the tryptophan substitutions relative to the ion-pore and possibly to the bilayer might be a critical factor in determining the degree of perturbation. To define a mechanism by which bulky aromatic replacements in the M4 transmembrane segment produce such an effect on the nAChR channel gating, it was imperative to identify which positions in this domain were capable of modulating ion channel gating. Subsequently, the mechanistic contribution of the M4 transmembrane segment to channel gating was examined using burst-oriented analysis at several acetylcholine concentrations (38). This study showed that the increase in the open channel probability of the αC418W mutant was due to a decrease in the channel closing rate and possibly an increase in the effective opening rate.

Another study demonstrated that replacement of αC418 and αT422 with alanine in the muscle-type nAChR increased the channel closing rate (39). Moreover, we have substituted the homologous C418 residue in the M4 domain of α subunit in the muscle-type nAChR for a variety of amino acids including, polar, hydrophobic, negatively and positively charged side chains (27). A total of nine amino acid replacements (G, A, V, L, S, M, W, H, and E) at this αC418 position were expressed on Xenopus oocytes and ion channel function was examined by whole-cell voltage clamp and patch clamp. From these nine mutants, only the αC418W shows a significant decrease in the EC_{50} for ACh, approximately 10-fold. In addition, the single channel data recorded for this mutation displays an increase in the mean channel open time of about 4.5-fold. The C418E and C418H, which possess charged side chains, did form functional nAChR channels. While
the C418E substitution may act as a hydrophobic anion, the C418H substitution is not so strongly charged. Previously, it was found that nACHR mutated to Arg at the αC418 position did not express on the oocyte surface (40). It is conceivable that the αC418H mutant may be able to insert into the membrane with its neutral form at physiological pH.

A remarkable observation was found in the mutant αC418G, it prevents the functional expression on muscle type nACHR (27) while in the Torpedo receptor, this mutant exhibited low expression levels (35). This variation in response might be due to structural modifications and/or differences in the interaction with the lipid environment between these receptor subtypes. There are four positions in the mouse αM4 that are different from Torpedo: G412, L419, L423 and A424. The αC418G mutation in the mouse αM4 produces a transmembrane segment containing three glycine residues: GVFMGVLGIGTVFA. In the Torpedo receptor the same mutation only contains two glycine residues: CVFVLMILGTVSA. The disruption of secondary structure elements in the mouse αC418G mutation is probably more drastic due to the presence of three glycine residues in a ten amino acid sequence. One of the rules that allow prediction of α-helix formation with 80% accuracy is that if a sequence of six residues contains two or more ‘helix breakers’, helix formation is unlikely (41). The introduction of a glycine residue in the mouse αC418 position could be this case, and this might be a possible cause for a lack of functional expression in this mutation. Although the introduction of an α-helix breaker at this αM4 position was lethal to the functional assembly of the muscle-type nACHR, it was difficult to make inferences on secondary structure elements of this lipid-exposed transmembrane segment based solely on these data.

In a subsequent study we introduced tryptophan substitutions at twelve positions (C412W, M415W, L416W, I417W, C418W, I419W, I420W, G421W, T422W, V423W, S424W, and V425W) along the α-M4 of the Torpedo nACHR (28). 125I-α-Bungarotoxin binding assays on the *Xenopus laevis* oocytes expressing wild type and mutant nACHR demonstrated that tryptophan residues can be accommodated along the entire M4 domain of the α subunit except at position I417. Since the I417W was the only mutant that did not express functional receptor in this study, it was speculated that this M4 position is critical for the nACHR folding, oligomerization and perhaps transport to the membrane. It is likely that I417 is in close contact with residues from other domains or closely packed to maintain the protein structure in the membrane. Mutations L416W, I419W and I420W showed a significant reduction in expression when compared to wild type. The reduced expression of these mutations suggested that the efficiency in receptor assembly or oligomerization was substantially reduced by the tryptophan substitution. Three lipid-exposed mutations (C412W, C418W and V425W) produced a gain in function response. A summary of the functional changes in nACHR expression and function are shown in Figure 3. EC50 values and nACHR expression levels (fmol) were plotted as a function of M4 sequence positions 415 through 425. The oscillation of the function that describes these parameters as a function of position 415 through 425 indicates a periodicity of about 3.6 amino acids (see bars in Figure 3). The later results provided additional experimental evidence of a potential α-helical structure for the Torpedo αM4 domain. Figure 2 illustrates an α helical diagram for α-M4. It is noteworthy that mutations that produced a gain in function response face the lipid interface,
Cholesterol and acetylcholine receptor

Figure 3. Periodicity of changes in nAChR expression and function plotted as a function of tryptophan substitution at positions 415 to 425 in the αM4 transmembrane segment. The scale shown in the bar indicates 3.6 amino acids. The dashed line indicates the wild type value for each parameter. The asterisk indicates the I417W mutation that did not produce a functional nAChR.

whereas inhibitory mutations are facing the interior of the protein, possibly facing M3 or M1. In summary, our tryptophan scan study, together with photolabeling studies using Torpedo membranes (31-32), suggest that the αM4 is an α-helix.

Cholesterol regulation of nAChR function in reconstituted membranes

The laboratories of McNamee and Barrantes were the first to describe the requirement of anionic and neutral lipids such as cholesterol to maintain functional nAChRs in reconstituted phosphatidylcholine membranes. This reconstitution system was used for over twenty years to study the interaction of nAChRs with the lipid environment (for review see 42-45). The first feature that emerged from the reconstituted nAChR studies was this receptor's sensitivity to the lipid environment (46-49). Furthermore, it was found that several lipid mixtures were able to fully support both ligand binding and ion channel functions, most notably those containing cholesterol and negatively charged phospholipids. Another remarkable observation was that many neutral lipids such as tocopherol, vitamins D3, K₁, and several cholesterol analogs could be substituted by cholesterol (50-51). These observations suggested that structure-function requirements for cholesterol are not very specific. Furthermore, cholesterol could not restore nAChR function in vesicles made from purified egg phosphatidylcholine (PC) (52). These studies confirmed that other phospholipids, i.e. dioleoylphosphatidylcholine (DOPA) may be required for cholesterol to exert its
modulatory effects. Since then several groups have performed biophysical studies using numerous techniques and preparations in order to define the interaction of nAChRs with specific lipids and to correlate nAChR function with the physical properties of the lipid environment. After two decades and despite a wealth of experimental data from reconstitution systems, the role of membrane cholesterol in regulating the nAChR function remains obscure.

Direct interaction of cholesterol and nAChRs

Possible cholesterol-nAChR interactions have been studied by several groups. Middlemas and Raftery (53) and have shown that all four subunits of the *Torpedo* receptor can be labeled by the photoreactive cholesterol analog [3H]-cholesteryl dyazoacetate. Following cholesterol depletion of *Torpedo californica* AChR-rich membranes, Leibel et al., (54) found the existence of two pools of cholesterol: one tightly bound to the nAChR-channel complex and another less tightly bound fraction, which was suggested to be associated with bulk lipid. Jones and McNamee (55) have demonstrated that cholesterol does not displace phospholipids from the protein-lipid interface. Cholesterol, however, may interact directly with the receptor at protein-protein interfaces or "non-annular" sites. This study lead to the proposal that there are five to ten non-annular sites for cholesterol, located in the interstices between the five subunits, and to the speculation that cholesterol's occupancy of these sites may have an activating effect on the nAChR function. Moreover, the results from this study demonstrated that a minimum of 45 lipids per receptor are required to prevent irreversible loss of ion channel function. Latter McNamee's group confirmed this interaction by labeling the lipid-exposed Cys 451 in the γ subunit M4 with a pyrene group and quenching the cysteine bound pyrene using brominated cholesterol hemisuccinate (56).

A work by Keith Miller's laboratory using *Torpedo nobiliana* expanded the hypothesis that cholesterol could bind non-annular sites in the nAChR (57). These investigators found that cholesterol analogs that prevent flipping across the membrane did not influence channel opening, leading to the conclusion that flip-flop is not a key part of the mechanism of nAChR activation. Furthermore, when nAChR was reconstituted with a cholesterol hemisuccinate that was attached to glycerol backbone of phosphatidylcholine (1-oleyl-2-(cholesterol hemisuccinyl) phosphatidylcholine), channel activation was still observed. They concluded that cholesterol binding sites must be close to the lipid-protein interface, however, they suggested that these sites were "perianular" based on the idea that this "tethered" cholesterol analog would not be buried within the nAChR subunits. According to their model, these "perianular" sites are located within the crevices of the surface of the protein that are in direct contact with the lipid bilayer, whereas, annular sites applies to the remaining area of the lipid-protein interface where lipid motion is restricted. They also proposed to classify the non-annular sites as interstitial sites.

Another photolabeling study by the group of González-Ros found that the cholesterol analog p-azidophenacyl3alpha-hydroxy-5beta-cholan24-ate (APL) binds all four subunits of the nAChR (58). More recently, the binding sites for cholesterol at the protein interface of the *Torpedo* nAChR were mapped using a novel photoreactive analog of cholesterol (125I-azido-cholesterol) (59). In contrast to the previous studies by Raftery and Gonzales-Ros groups, this study found that cholesterol-binding sites were
exclusive to the $\alpha$ M4 and M1 and $\gamma$ M4 transmembrane segments. Furthermore, they found that the labeling pattern by $^{125}$I-azido-cholesterol was the same in the presence or absence of agonist. These investigators suggested that the nAChR does not have a significant change in surface area during agonist activation leading to the proposal that cholesterol might acts as a “molecular grease” that facilitated the movement of transmembrane segment during conformational transitions.

The binding of lipids to the proposed non-annular sites (interstitial sites) should be considered in a wider context, for example, in facilitating the insertion of membrane proteins into lipid bilayer. This concept is consistent with the success of detergents containing a steroid nucleus, that is CHAPS and cholate in reconstituting multisubunit membrane proteins due to the stabilization of the protein structure during solubilization.

**Effect of cholesterol on the affinity state transitions of the nAChRs and ion channel function**

Cholesterol and other sterols have been reported to affect several aspects of the nAChR function including allosteric binding transitions and ion channel function. Initially, it was suggested that cholesterol was an absolute requirement for allosteric transitions (60). However, subsequent work demonstrated that several lipids and lipid mixtures could facilitate allosteric transitions even in the absence of cholesterol (48). The major effect of cholesterol described in the reconstituted nAChR was on ion-channel function. For example, an enhancement of agonist-induced cation flux by cholesterol was demonstrated in reconstituted vesicles depending on the type and composition of other lipids present (48; 60-62). These studies demonstrated marked cholesterol-dependent changes in ion gating of reconstituted nAChR-rich vesicles, however, the explanations on the magnitude and nature of this effect were not clear. The role of cholesterol in slow desensitization is also unknown, however, one study suggested that desensitization depends only on the prolonged exposure to agonists and does not depend on any other lipid including cholesterol (52). The binding kinetics of ethidium bromide to the reconstituted *Torpedo nobiliana* nAChR suggests that, in the absence of cholesterol, the receptor is “locked” in the R (resting) conformation (63-64). Rankin and co-workers (63) showed that the number of nAChR that open in response to high agonist concentration increase with the percentage of cholesterol in the bilayer, reaching a maximum at cholesterol molar percent of 30.

**Effect of cholesterol on the secondary structure of the nAChR**

Another possibility to be considered is that cholesterol affects the secondary structure of nAChRs. The first reported study using FTIR to assess the secondary structure of *Torpedo nobiliana* was performed by Moore et al., (65) and reported that the receptor has 34% $\alpha$-helices and 29 % $\beta$-sheets. McNamee’s group performed FTIR to analyze the effect of lipids that support ion channel function on the structure of the nAChR. The first study by Fong and McNamee (66) found that when both sterol and negatively charged phospholipids were present in the membranes, both $\alpha$-helical and $\beta$-sheet contents increase. That is, $\alpha$-helices are stabilized in membranes containing cholesterol while $\beta$-sheets are stabilized in membranes containing negatively charged phospholipids. Interestingly, this data is consistent with the up-dated structure of the nAChR; were sterol facilitates ion channel function given that all transmembrane
segments have been recently proposed to be α-helical structure (23). The stabilization of beta structure by negatively charged phospholipids, as proposed by McNamee's group, is crucial in transmitting conformational changes induced by nAChR agonists. This is consistent with a recent work by Steve Sine's group in which a lysine-scanning of the ligand-binding domain of the nAChR suggest a β-sheet structure (67). Another FTIR study using hydrogen/deuterium exchange in the presence and absence of carbamylcholine did not found large net changes in secondary structure suggesting that the nAChR desensitization does not result from a large perturbation of the secondary structure (23). In contrast to McNamee's results the aforementioned study found that the spectral features of the nAChR in the presence and absence of cholesterol were very similar, thus suggesting that cholesterol was not a major regulator of the nAChR. In a later study, Méthot and Baenziger (24) found that, subsequent to exposure of the nAChR with $^2$H$_2$O, the infrared spectra recorded as a function of time revealed a strong intensity of the unexchanged α-helical vibration. This was interpreted to originate from the transmembrane segments.

Membrane fluidity and nAChR function

In 1986, work by Fong and McNamee suggested that cholesterol regulation of membrane fluidity plays a key role in its modulation of AChR function. It is noteworthy that other studies have challenged the membrane fluidity hypothesis (50; 52). The hypothesis that membrane physical properties influence nAChR function was proposed again by the group of Baenziger in Canada (23-25; 68). These studies proposed that membrane fluidity modulates the population of nAChRs in the resting and desensitized states and that the presence of anionic phospholipids are essential to stabilize fully functional nAChRs. The recent study by daCosta et al. (68) further evaluated the hypothesis that physical properties of the lipid modulate nAChR conformational transitions (68). They found that incorporation of nAChRs into phosphatidic acid containing membranes leads to dramatic increases in both, lateral packing densities and the gel to liquid crystal phase transition temperatures of the reconstituted membranes. These results prompted the hypothesis that a unique coupling between nAChR and the physical state of the phosphatidic acid containing membrane could be important to the modulation of nAChR function.

Cholesterol regulation of nAChR function in intact cells

Lipid-protein interactions of the nAChR have been extensively studied using reconstituted membranes (43; 55; 66; 69-72). Several efforts have been directed to understand the nature of these interactions but the mechanism responsible for the modulation of nAChR by membrane lipids, especially cholesterol, remains to be elucidated.

In 1983, Young and Poo performed pioneer experiments in Xenopus myotomal muscle cells, suggesting that the nAChR function depends on its molecular environment in Xenopus myotomal muscle cells. They applied a uniform electric field to rearrange receptors and to form nAChR clusters. Their results showed that regions with nAChR clusters displayed an increase in open channel time when compared to non-clustered receptor regions, thus suggesting that the function of the nAChR depends on its molecular environment (73). In 1986, Lechleitner and colleagues examined the effects of
halothane in cholesterol enriched and depleted *Xenopus* myocytes transfected with nAChR. They used cholesterol-rich liposomes to increase the cholesterol to phospholipid (C/P) molar ratios of myocytes from 0.16 to 0.57 and cholesterol-free liposomes to decrease C/P molar ratios from 0.16 to 0.10. Cholesterol enrichment attenuated the halothane-induced reduction of the nAChR burst duration; while in cholesterol-depleted myocytes, halothane enhanced reduction of the channel burst duration, suggesting that the lipid environment affects the modulation of nAChR function. The authors concluded that cholesterol causes a perturbation in the membrane matrix, suggesting that general anesthetics interact indirectly with nAChR through lipid domains (74).

Almost ten years later, Lasalde, et al. (75), used cholesterol-rich liposomes to increase cholesterol concentrations of chick myocytes expressing nAChR. The C/P molar ratios of the myocytes were altered form 0.24 to 0.52. Cholesterol-enriched myocytes showed two channel conductances, 56pS and 36pS, while the control only showed a 50pS conductance; suggesting that cholesterol-enriched myocytes had two different types of nAChR conformational states. Of these, the 36 pS channel conformation represents an inhibited one and reflects the major possible membrane perturbation caused by cholesterol. Two hypotheses were proposed by the authors, the first was that cholesterol induces phospholipid segregation producing a heterogeneous distribution of the nAChR in the lipid bilayer and the second that there was a direct interaction of the sterol with the nAChR at high cholesterol levels (75).

Barrantes and coworkers have studied the influence of cell membrane environment and physical state on the function of the muscle nAChR. To achieve this, they used the lipophilic probe laurdan, which gives a measure of gel and/or liquid-crystalline phospholipid phase qualities of the membrane in which it is inserted. They demonstrated that ion permeation and channel kinetics do not depend only on the nAChR subunit composition but also on the lipid environment and the physical state of the lipid environment surrounding the protein (76).

In summary, these studies demonstrate that lipid manipulations in intact cells are achievable and are a useful tool in the study of lipid-protein interactions. Moreover, they have provided evidence supporting the notion that alterations in the membrane lipid environment have a significant effect on nAChR function. Additional studies are needed to answer two crucial questions. What is the role of the protein-lipid interface in receptor function? And which are the mechanisms of action of lipids as modulators of nAChR?

**Cholesterol regulation of nAChR function in *Xenopus* oocytes**

Recently, our laboratory examined the effects of cholesterol enrichment and depletion on nAChR function in *Xenopus* oocytes (77). The C/P molar ratio in the oocyte membrane was increased from 0.5 to 0.87 using cholesterol-rich liposomes. We used the fluorescent cholesterol analog (22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-y1)amino)-23,24-bisnor-5-cholen-3-ol) to monitor cholesterol incorporation into the surface membrane of the oocytes using confocal microscopy, which allowed us to estimate the approximate time of liposome exposure required to reach a C/P molar ratio of 0.87 (Figure 4). Figure 5 shows the main protocols used in our experiments; neither cholesterol enrichment nor depletion severely affected the resting membrane potential of the oocytes.
Figure 4. Confocal imaging of cholesterol enrichment in the plasma membrane of *Xenopus* oocytes. Image reconstruction of 32 sequential confocal images showing incorporation of a fluorescent cholesterol analog (22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24- bisnor-5-cholen-3-ol) to the surface of a *Xenopus* oocyte. Liposomes containing the fluorescent cholesterol analog (inside yellow circles) are shown fusing with the plasma membrane (left). In the insert, 4 of a total of 32 sections are shown (right). Images were acquired using a BioRad MRC 600 confocal microscope. Confocal sections were taken at intervals of 10 μm.

Figure 5. Methods for cholesterol manipulations in *Xenopus* oocytes. The oocytes were perfused with 100 μM of Acetylcholine for the initial current measurement. After that the oocytes were incubated with cholesterol-enriched liposomes or β-methyl cyclodextrin to obtain a moderated enrichment or depletion of the cholesterol levels respectively. The oocytes were washed to remove the excess of cholesterol-enriched liposomes or β-methyl cyclodextrin and perfused again with 100 μM of Acetylcholine for the second current measurement.
Cholesterol enrichment reduced the wild type macroscopic response to acetylcholine by 52%, while the reduction for the C418W mutant was 81% (Figure 6), suggesting that the functional response of the C418W is more sensitive to high cholesterol levels than wild type. The EC₅₀'s for ACh are similar after enrichment, thus suggesting that the loss in functional response could be due to a reduction in the number of resting nAChR rather than an inhibitory effect on the ion-channel function. Depletion of endogenous cholesterol from oocytes membranes was performed using methyl-β-cyclodextrin to reach C/P molar ratios of 0.30. Oocytes expressing the wild type receptor had a net increase of 4.64% on the macroscopic response after cholesterol depletion; in contrast the C418W mutant showed an increase of 67% in the macroscopic response, suggesting that this mutation displayed a significant gain in function response and alter the receptor modulation by cholesterol (Figure 7). Several questions emerged from these results: Why would a lipid-exposed mutation produce an activation after cholesterol depletion? Which types of interactions govern this increase in response? Does cholesterol depletion causes an increase in the open channel probability of the αC418W or an increase in the active pool of nAChR in the plasma membrane?

The action of cholesterol on the nAChR has been associated with the bulk-lipid bilayer fluidity (25; 48; 50). It has been suggested that the membrane fluidity modulates the populations of nAChR in the resting (R) to desensitized states (D); where the receptor

![Figure 6. Cholesterol enrichment produces an inhibitory response of the nAChR.](image)

A and C panels show current traces for the wild type (WT) and the mutant αC418W, before and after cholesterol enrichment. The panels B and D show the average percentages of inhibition of WT and αC418W nAChR after cholesterol enrichment. There is a 51.5% of inhibition of WT (n = 20) and 80.5% on the αC418W (n = 16). In both cases the controls are oocytes that were incubated with MOR2 for 45 minutes instead of the cholesterol enriched liposome.
Figure 7. Cholesterol depletion produces an increase of the macroscopic response. Oocytes expressing wild type AChR (A and B), the average current after cholesterol depletion was increased by 6.6% estimated from the difference between the experimental and control (cholesterol depleted 8.6% - 2.0% control; n = 11) (E). In oocytes expressing the αC418W AChR (C and D), the average current after cholesterol depletion was 66% (100% cholesterol depleted - 34% control; n = 22) (F).

is on its resting state at lower membrane fluidity and an increase in membrane fluidity leads to a shift towards the desensitized state (25). The functional response of the Torpedo wild type to alterations in membrane cholesterol levels might look like a bell-shaped profile and the mutation αC418W displaces this bell-shaped profile to higher fluidity levels (Figure 8). We observed a higher degree of activation with higher membrane fluidity, which might be caused by a gain in function response and/or an increase in the resting pool of nAChR (77). The tryptophan residue at position αC418 could be altering normal interactions of the nAChR with cholesterol leading to the observed increase in sensitivity to membrane cholesterol levels. A possible explanation for these results is that the opening transition of the αC418W is facilitated in a more fluid environment due to penetration of the tryptophan side-chain in the bilayer, leading to a more stable open-channel state. Alternatively, the equilibrium between resting and desensitized states of the nAChR is very sensitive to cholesterol levels in the membrane, and higher cholesterol levels in both cases decrease the active pool of receptors (77).

Our studies with cholesterol and nAChR suggest that the mechanism by which cholesterol modulates the receptor function might involve specific structural interactions or perhaps specific orientations of the transmembrane domains that are exposed towards
the lipid environment. Kinetical analysis after membrane lipids manipulations will be required to fully understand the mechanisms that govern the nAChR and its modulation by membrane lipid composition.

**Imaging applications to visualize nAChR, lipids and cholesterol in cell surfaces**

Most of the studies using nAChR have been directed to understand its structure and kinetics (for review see: 3; 78-79). The efforts directed to understand lipid-protein interactions of the nAChR have mostly used the *Torpedo* receptor in reconstituted membranes (43; 55; 66; 69-72). These efforts have been successfully extended to intact cells (74-77). Given that lipid manipulations in intact cells are feasible, the next logical step to better understand the nature of lipid-protein interactions should involve imaging studies.

*Xenopus* oocytes are a robust expression system for *Torpedo* nAChR and can thus be used to study lipid-protein interactions of this receptor. One advantage of using the *Xenopus* expression system is the use of the nonsense suppressor method (80); which will permit to tag and visualize specific positions along the lipid interface. Moreover, it is possible to quantify its major classes of membrane carbohydrates, phospholipids and proteins as well as to estimate its membrane cholesterol to phospholipid molar ratio (77; 81-82). Drawbacks of using oocytes, however, are the large quantity of yolk platelets and their fragility. Because confocal microscopy allows for optical sectioning of the specimen of interest, it eliminates the need for unnecessary manipulation and diminishes autofluorescence from yolk platelets (83).

The majority of the imaging studies of the nAChR have been designed to understand protein-protein interactions of the receptor at the neuromuscular junction and in the *Torpedo* electrocyte (reviewed in: 84). In 1990, Froehner and colleagues used fluorescence microscopy to study protein-protein interactions of the nAChR expressed in
Xenopus oocytes. They wanted to test the hypothesis that a 43kDa protein, rapsyn, has a role in the formation of clusters of nAChR. In this study oocytes were cut, mounted onto slides and flattened, leaving the membrane free of autofluorescence from yolk platelets. The authors observed that the mouse nAChR are uniformly distributed in the surface of the membrane, yet when oocytes were co-injected with mRNA encoding for a 43kDa protein, nAChR clusters of 0.5-1.5μm in diameter appeared, thus suggesting that this 43kDa protein induces membrane clustering of the nAChR (85). In 1994, Wheeler’s group expressed nAChR in Xenopus oocytes and used confocal microscopy to examine the role of the β-subunit on the 43kDa protein-induced clustering of the nAChR. They found that replacing muscle β-subunit with neuronal β2 subunit prevented the 43kDa protein-induced clustering of the nAChR, thus suggesting that this subunit has an important role in receptor localization (86).

To our understanding, hardly any study in the literature has used oocytes as a system to visualize lipid-protein interactions. The effects of lipid composition on the regulation of the nAChR can be assessed using fluorescently labeled alpha bungarotoxin and fluorescent markers of cell membrane lipids. Recently, Brusés and colleagues labeled the α7-nAChR with α-bungarotoxin and cholesterol-rich lipid microdomains (lipid rafts) with fluorescein-conjugated cholera toxin (FITC-CTX). Using confocal microscopy they were able to quantify fluorescence intensity, fluorescence intensity ratios and measure receptor clusters number and size. They found co-localization of lipid rafts and the α7-nAChR during synaptogenesis of somatic spines of ciliary neurons. Moreover, these investigators found that cholesterol depletion in ciliary neurons induces the dispersion of lipid rafts and causes the redistribution of α7nAChR into smaller clusters. The latter results demonstrate that the integrity of cholesterol-rich domains in the membrane is required to maintain α7-nAChR clustering (87) and are consistent with the idea of a functional relationship between membrane cholesterol and the α7-nAChR.

Visualizing cholesterol-nAChR interactions using imaging techniques is an important issue that, to our knowledge, has not been addressed. To study those interactions, suitable cholesterol fluorescent analogues and nAChR fluorescent markers must be used simultaneously. To design a fluorescent cholesterol analog that faithfully emulates cholesterol has proven a difficult task (88). Such fluorescent molecules often have a bulky fluorescent group that may interfere with cholesterol’s positioning and function in the fluid lipid bilayer. The cholesterol molecule consists of a steroid ring system in the middle, an alkyl tail on one end and a 3β-hydroxyl on the other end. The hydroxyl group is of particular importance as it holds the cholesterol molecule at the membrane interface (88-89). Several requirements must be met in the design of a biologically active cholesterol analogue. Such molecule must have a free 3β-OH end, a branched seven-carbon alkyl chain at the 17β-position, an unaltered alicyclic chain, a planar Δ5(6) double bond and angular methyl groups (88; 90). Dehydroergosterol (DHE) meets the above criteria, its only difference being having an extra methyl group and three additional double bonds. Two of these double bonds and the Δ5(6) double bond are believed to confer fluorescence to DHE (88). Studies have demonstrated that DHE has biological activity that closely resembles that of cholesterol (91). The main drawback of using DHE resides in its excitation spectrum as this molecule excites in the UV region (310-338 nm) where it undergoes severe photobleaching (92). Photobleaching can be diminished using a multiphoton laser (usually infrared laser) to excite the fluorophore of
interest. Such laser will only excite the fluorophore at the focal plane, thus eliminating fluorophore excitation above and below the focal plane. Moreover, infrared lasers penetrate biological tissue much better than UV lasers and this makes it possible to excite any given fluorophore with less laser intensity, which also diminishes photobleaching. In a recent study, Frolov et al., (92) successfully employed DHE and multiphoton microscopy to study different aspects of cholesterol metabolism. Efforts are underway in our laboratory to use multiphoton microscopy in the study of cholesterol-nAChR interactions.

Final remarks

Based on the data gained from reconstitution experiments three mechanisms for cholesterol regulation of nAChR function have been proposed: (1) cholesterol can interact at some discrete class of binding sites on the receptor to affect the conformational state of the receptor, (2) cholesterol can influence the nAChR function indirectly by altering the biophysical properties of the lipid membrane and (3) cholesterol may act as a “molecular grease” that facilitates the movement of transmembrane segments during conformational transitions of the nAChR. The first mechanism involves a direct molecular interaction of cholesterol with the nAChR, while the second has been associated to a more complex scenario that involves cholesterol regulation of membrane fluidity playing a key role in the modulation of nAChR function. The third mechanism was suggested based on the observation that the nAChR does not have a significant change in surface area during agonist activation, however, it also involves a direct interaction of the sterol with the nAChR. Despite many years of study, the functional relationship between cholesterol and nAChR function remains unclear perhaps due to paucity in studies that could provide a more comprehensive picture of the dynamics of nAChRs in an intact cell membrane. In order to evaluate and test the models gained from decades of work in the reconstituted system it is necessary to move on to more dynamic experimental systems.

Few studies have used natural membranes to assess the role of cholesterol on nAChR. The use of recombinant nAChR expressed in oocytes or cell lines must be further explored given that they provide a powerful system to assess the role of the lipid-protein interface on nAChR function. Taking the information gained from the mapping of key lipid-exposed residues, it will be possible to assess the effect of cholesterol on lipid-exposed mutations using electrophysiological recordings. Finally, caution must be exercised when drawing conclusions on how cholesterol affects the nAChR function. Obviously, Torpedo is the first model to be examined because it can be consolidated with data gained from reconstitution studies. However, by no means the results obtained from the Torpedo nAChR should be extrapolated to other nAChR species such as muscle or neuronal subtypes, given that these receptors are structurally and functionally different.

The use of confocal microscopy will aid in the understanding of cholesterol-nAChR interactions. Double labeling of nAChR and lipids in intact cell membranes will help understand the nature of these interactions. This approach needs to be further developed and the appropriate fluorescent cholesterol analogs must be used since the addition of fluorescent reporter groups may interfere with cholesterol’s positioning and function in the fluid lipid bilayer. The development of a novel method to visualize lipid-protein
interactions of the nAChR *in vivo* will be an innovative mean to study the structural and functional aspects of lipid regulation of the nAChR that is related to several pathologies of the neuromuscular junction and the human brain.

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**References**