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Heterogeneous distribution of acetylcholine receptors in chick myocytes induced by cholesterol enrichment

Jose A. Lasalde *, Antonio Colom [1], Edgard Resto [2], Conchita Zuazaga

Instituto de Neurobiologia, Universidad de Puerto Rico, Recinto de Ciencias Medicas, Boulevard del Valle 201, San Juan, PR 00901, USA

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Abstract

The cholesterol concentration at the cell surface of cultured chick myocytes was increased in order to determine the effects of high levels of cholesterol on the ion channel properties of the nicotinic acetylcholine receptor. Single channel recordings and fluorescence polarization studies using 1,6-diphenyl-1,3,5-hexatriene (DPH) were performed under equivalent conditions for normal and cholesterol enriched myocytes. In cell attached patches from myocytes with a cholesterol to phospholipid molar ratio (c/p) of 0.24 and a microviscosity of 1.35 poise a single conductance of 51 pS was detected. The cholesterol enriched myocytes with a c/p of 0.52 and a microviscosity of 2.05 poise showed two conductances, a 54 pS and a 39 pS channel: both were blocked by α-bungarotoxin. The 39 pS channel was detected with the simultaneous appearance of a slow component of τ_m (modulation time) for DPH fluorescence measured by phase demodulation. The 80% reduction in the open time constant (τ_2) of the 39 pS channel suggest an inhibition of the normal conformational state. The combined results suggest that cholesterol enrichment may induced a more heterogeneous lipid environment and that the two types of channel properties could result from the distribution of the receptors in different domains.

Keywords: Acetylcholine receptor; Single-channel current; Fluorescence anisotropy; Cholesterol enrichment

1. Introduction

The role of membrane cholesterol on the nicotinic acetylcholine receptor (AChR), a ligand-gated and cation-selective ion channel protein, has been the subject of extensive research during the last decade. The fact that the structural and functional features of AChR have been extensively characterized make this membrane protein one of the best experimental models to study lipid–protein interactions. Several studies have shown that AChR function is sensitive to the lipid environment [1–3]. In this respect it has been shown that the AChR activity could be maintained in the presence of certain lipid mixtures, especially those containing cholesterol and negatively charged phospholipids [3]. Subsequent work has been directed towards correlating the degree of interaction of the AChR receptor-channel function with the physical properties of specific lipids [4]. Middlemas and Raftery [5] have shown that all four subunits can be labeled by a photoreactive cholesterol analog. Following cholesterol depletion of *Torpedo californica* AChR-rich membranes, Leibel et al. [6] found the existence of two pools of cholesterol: a tightly bound fraction that presumably was associated with the AChR-channel complex and another less bound fraction which was suggested to be associated with bulk lipid. Using single channel recordings in *Xenopus laevis* myocytes, Lechleiter et al. [7] reported that the alteration in channel kinetics elicited by the presence of halothane was attenuated when membrane cholesterol was increased, and enhanced when membrane cholesterol was decreased. These results were interpreted as a competitive effect of cholesterol with halothane, which was proposed to be acting at the lipid/receptor interface. In a fluorescence quenching study, Jones and McNamee [4] found that

Abbreviations: AChR, acetylcholine receptor; BuTx, α-bungarotoxin; ^{125}I-BuTx, ^{125}I-labeled BuTx; DPH, diphenylhexatriene; ACh, acetylcholine; τ_m, modulation time; τ_p, phase time; PC, phosphatidylcholine.

* Corresponding author. Present address: Section of Molecular and Cellular Biology, Division of Biological Sciences, University of California, Davis, CA 95616, USA. E-mail: jlasalde@ucdavis.edu. Fax: +1 (916) 7523085.

[1] Present address: Departamento de Quimica, Universidad Interamericana, Recinto Metropolitano, Rio Piedras, PR 00919, USA.

[2] Present address: Turabo University, Ana G. Mendez Educational Foundation, Gurabo, PR 00658, USA.

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cholesterol was able interact directly with the *Torpedo* AChR at sites which supposedly represent protein/protein interfaces.

Despite a wealth of experimental data from reconstituted systems, the role of membrane cholesterol in regulating the AChR function has not been elucidated and somewhat controversial results have been obtained. For example, some studies suggest that the affinity state transition of the receptor is independent of the presence of cholesterol [3,8]; however, other studies have demonstrated that cholesterol is essential in maintaining this transition [9]. Although marked cholesterol-dependent changes in the ion gating event of reconstituted AChR-rich vesicles have been repeatedly shown [2,3,8] the explanations on the magnitude and nature [10] of this effect are still not clear. In addition, the role of cholesterol in AChR desensitization is also unknown. The reconstitution system itself and the variety of experimental techniques used has been suggested as the possible cause of this lack of agreement [6].

In this study we have increased the cholesterol concentration at the cell surface of cultured chick myocytes in an attempt to induce artificially high levels of cholesterol in the plasma membrane. We have examined the physical state of the plasma membranes of cultured chick myocytes by the use of fluorescence polarization methods under conditions equivalent to those used for single channel recordings of AChR channel activity.

2. Materials and methods

2.1. Tissue culture

Pectoral muscles from 10–12-day-old chick embryos were dissected, dissociated and plated either on 35 mm petri dishes for patch clamp experiments, or on 1 cm × 4 cm glass at a density of 10^6 cells/ml for fluorescence measurements. The cells were grown in a medium containing Dulbecco's modified Eagle's medium (DMEM, Gibco), 10% horse serum, 2% chick embryo extract, 0.5% penicillin and 0.5% streptomycin. The cells were grown at 95% relative humidity in a 5% CO_2/95% air atmosphere. After 48 h in culture 10^{-6} M cytosine arabinose was added to reduce fibroblasts growth. Lipid modifications were performed 10–15 h before patch clamp recordings.

2.2. Cholesterol loading of myocytes

This modification was done using two methods. The first was performed as described by Horwitz et al. [11]. Cholesterol loading was carried out by direct addition of cholesterol to the medium using a stock solution made up in ethanol. The final cholesterol concentration in the medium was 5 μg/ml (13 μM) while the cholesterol concentration in the control medium was 1.2 μM. The second method used to load and also deplete cholesterol levels in these myocytes was the liposome incubation technique [12]. Liposomes were diluted to 15% in DMEM just prior to use and added to myocytes cultures for a period not exceeding 14 h. A fresh liposome suspension was replaced every 3 h during the incubation period.

2.3. Preparation of liposomes

Egg phosphatidylcholine (80.0 mg) (Avanti Polar Lipids, Birmingham, AL) was mixed with cholesterol (40.0 mg) (Calbiochem, La Jolla, CA) in chloroform, dried under vacuum, resuspended in 10 ml of DMEM and sonicated using a Bransonic-12 sonicator under nitrogen at 4° C (1 min on, followed by 1 min off, for 80 min). The lipid suspension was clarified by centrifugation (11 500 rpm, 30 min, 4° C). The supernatant containing unilamellar liposomes was filtered (Millipore, 0.2 mm) and stored at 4° C.

2.4. Cholesterol to phospholipid molar ratio determination

The lipid extraction from the plasma membrane fraction was based on the procedure described by Folch et al. [13] with some modifications. Briefly, the extraction was done using a 8:4:3, chloroform/methanol/plasma membrane suspension. After removing the organic phase, the water phase was re-extracted with 1:2 methanol/chloroform. The two organic phases were combined, evaporated under reduced pressure, redissolved in 1:2 methanol/chloroform under nitrogen, and assayed for total phospholipid by phosphate analysis according to the method of Johnson [14]. Cholesterol oxidase assays were employed to quantitate total cholesterol [15].

2.5. Plasma membrane fraction preparation

50 plates of cells were scraped and washed with two volumes of an ice cold (4° C) isotonic phosphate buffered saline (PBS). The cells were harvested by centrifugation at 6000 rpm for 10 min, resuspended and washed in the same buffer. The pellet from the previous step was washed and then resuspended in 3 volumes of 10 mM Tris, 135 mM NaCl, pH 7.5. This membrane suspension was sonically disrupted at 4° C for 10–30 s periods. This fraction is the crude cell membrane extract. The crude cell membrane extract was diluted 1:1 with 20 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4 (Tris/Mg^{2+}/Ca^{2+}) buffer. The mixture was then directly chromatographed on an affinity column of approx. 2 ml of Concanavalin A-agarose (Sigma) that was previously equilibrated with the same buffer and maintained at 4° C. The column was washed with buffer at a flow rate of approx. 4 ml/h and 1 ml fractions were collected. An elution profile was constructed by measuring the absorbance of each fraction at 280 nm in a Perkin Elmer 552 spectrophotometer. Elution with starting buffer was maintained until the absorbance reached 0.005 units continually. The elution of Concanavalin A-binding plasma

membrane was accomplished with 20 mM β-alanine buffer at pH 3.7. After elution each fraction was adjusted to a pH of 7.5. All the eluted fractions were assayed for the presence of AChR using an α-bungarotoxin assay.

2.6. α-Bungarotoxin binding assay

The general binding procedure of ^{125}I-α-bungarotoxin (^{125}I-α-BuTx) to the plasma was carried out as follows: 10 nmol ^{125}I-α-BuTx were added to 50 ml from each eluted fraction and combined with 200 ml of 50 mM Tris, 1 mM $CaCl_2$, pH 7.4 and incubated for 30 min at 37° C. After incubation, the mixture was applied to microcolumn containing 100 ml of Concanavalin A-agarose packing previously equilibrated as described. Columns were washed with 3.0 ml of Tris Mg^{2+}/Ca^{2+} buffer and the microcolumn was counted for ^{125}I. As an alternate method, 200 ml of the Con A-agarose (50%) suspension was added to the reaction mixture immediately after the incubation period. The free ^{125}I-α-BuTx was washed from the Con A-agarose suspension by centrifugation at $12000 \times g$ for 2 min, and the supernatant was collected. The suspension was washed again with 200 ml of Tris/Mg^{2+}/Ca^{2+} buffer and the supernatant of this step was combined with the one from the first step. Gamma counting was performed in the supernatants and in the Con A suspension in a Beckman model 500 apparatus. The final elution profile was constructed via determination of the absorbance and counts on each fraction.

2.7. DPH-labelling

Diphenylhexatriene was dissolved in tetrahydrofuran at a concentration of $2 \cdot 10^{-3}$ M. Prior to use, it was diluted 1:2000 in phosphate-buffered saline (PBS) with vigorous mixing. Myocytes attached to the glass were washed three times with PBS and then exposed for 15 min to the labeling solution. Immediately after the period, the glass with the DPH-labelled myocytes was washed with PBS three times and loaded inside the cuvet to perform the fluorescence measurements. The incorporation of DPH into the membranes was followed by a steep increase in the fluorescence intensity.

2.8. Fluorescence measurements

All fluorescence data were obtained on a SLM 4800S (SLM Instruments, Urbana, IL) phase modulation spectrofluorimeter. Excitation was accomplished by using a 450-W Xenon arc lamp. For the steady-state polarization measurements, the excitation and emission monochromators were set to 365 nm and 420 nm, respectively. A bandpass of 4 nm was used for excitation and detection of fluorescence. For lifetime measurements, the samples were excited at 365 nm and a W6389 filter was placed between the sample and the emission detector. The intensity of the exciting light was modulated sinusoidally at a fixed frequency of 18 MHz with a Debye-Sears ultrasonic modulator. Fluorescence polarization data were obtained by measuring $I_{\parallel}$ and $I_{\perp}$ (where $I_{\parallel}$ and $I_{\perp}$ are fluorescence intensities detected through polarizers oriented parallel and perpendicular to the direction of polarization of the excitation beam). The steady-state anisotropy, r, is defined as:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} = \frac{R+1}{R+2}$$

where:

$$R = \frac{I_{\parallel}}{I_{\perp}}$$

From the experimental values of anisotropy, r, the microviscosity was determined using the following approximate expression:

$$\overline{\eta} = \frac{I_{\parallel}/I_{\perp} - 1}{0.73 - 0.27 \cdot I_{\parallel}/I_{\perp}} = \frac{2.4r}{0.362 - r}$$

where

$$r = I_{\parallel} - I_{\perp}$$

This expression can be used for an approximate evaluation of η ($\pm 15\%$) of systems labeled with DPH [16,17].

2.9. Patch clamp recordings and analysis

All the single channel currents were recorded using the on-cell attached patch configuration. Patch pipettes were made from soft glass tubing (haematocrit, Blue-Tip, Fisher Scientific) in two stages using a vertical puller (David Kopf, CA). When filled, the pipettes had resistances in the range of 7–12 MΩ, indicating tip diameters of approx. 1 μM. The pipettes were coated with Silgard (Dow Chemical, Midland, MI) to reduce background noise and fire polished to improve seal formation. The recording (pipette) solution was ACh 0.10 μM (Sigma) in a saline containing (in mM): NaCl, 140; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; Na-Hepes, 10; pH 7.4. Just before recording the currents the growth medium of chick pectoral muscle cells was replaced with the external solution (same as pipette solution without ACh). A Dagan 8900 patch clamp amplifier (Dagan, MN USA) with a 50 GΩ feedback resistor was used to amplify the channel currents. The channel activity in the cell-attached configuration at room temperature (20° C) was filtered at 10 kHz (−3 dB, 8-pole Bessel filter) and stored on a digital data recorder. For acquisition and detection the data was played back into a 386-based computer as an analog signal with redigitization at 4 kHz and sampled at 12 bit resolution every 50 μs using a Tecmar Labmaster analog-to-digital interface (Axon Instruments). The Fetchex routine from PCLAMP 5.5 (Axon Instruments, CA) was used for acquisition and single channel currents were detected with half-amplitude cross-

ing algorithm using IPROC 3 [18]. Single channel conductances were calculated from the slope of the current–voltage curve. Open time distributions were calculated from steady state recordings at −90.0 mV and fitted with two exponentials with the PSTAT routine (PCLAMP 5.5).

3. Results

The experimental evidence for the cholesterol enrichment of chick myocytes in culture was obtained using two methods. The first was by direct estimation of cholesterol to phospholipid molar ratios (c/p) for the isolated plasma membrane fraction. The second method was indirect and involved estimation of the membrane microviscosity (η) using 1,6-diphenylhexatriene (DPH), which is preferentially located in the plasma membrane of these cells [17]. Table 1 shows a correlation between c/p for the plasma membrane fraction and microviscosity for normal and cholesterol enriched myocytes. The microviscosity of the modified myocytes was considerably higher (52–61%) relative to the control cells. Cholesterol incorporation was also correlated with an increase in the DPH lifetimes in the modified cells. Differences in DPH lifetimes between the control and the cholesterol enriched myocytes are shown in Table 2. In the control myocytes there was some difficulty in measuring lifetimes by the modulation method. This suggested the presence of a very fast lifetime component(s) not detectable at this modulation frequency. In

Table 1
A correlation between c/p molar ratios from plasma fractions and membrane is shown

Sample	C/P (plasma fraction)	η (poise)
Normal myocyte	0.21–0.27	1.35 ± 0.19
Cholesterol enriched myocyte	0.47–0.58	2.05 ± 0.26

The surface membrane of myocytes 8–9 days in culture was exposed to a cholesterol enriched medium. After 15 h the modified myocytes showed a significant increase in microviscosity (η). The estimated c/p molar ratios from the plasma fraction was directly proportional to the microviscosity estimation.
η average from seven experiments.
c/p average from four experiments (see Materials and methods).

Table 2
A correlation of DPH lifetimes between the normal and cholesterol-enriched myocytes is shown

Experiment	Control		Cholesterol-enriched	
	τ_p	τ_m	τ_p	$\tau_{m(ns)}$
1	0.176	n.d.	0.975	5.49
2	5.644	n.d.	0.494	2.88
3	1.020	n.d.	1.499	5.27
4	−0.219	n.d.	0.966	3.43
5	0.198	n.d.	0.439	3.34

In the control myocytes there was some difficulty in attaining the modulation lifetime (τ_m). In the cholesterol enriched myocytes there are two major observations: (a) the τ_m became detectable, and (b) τ_m was always higher than τ_p.
n.d., not determined.

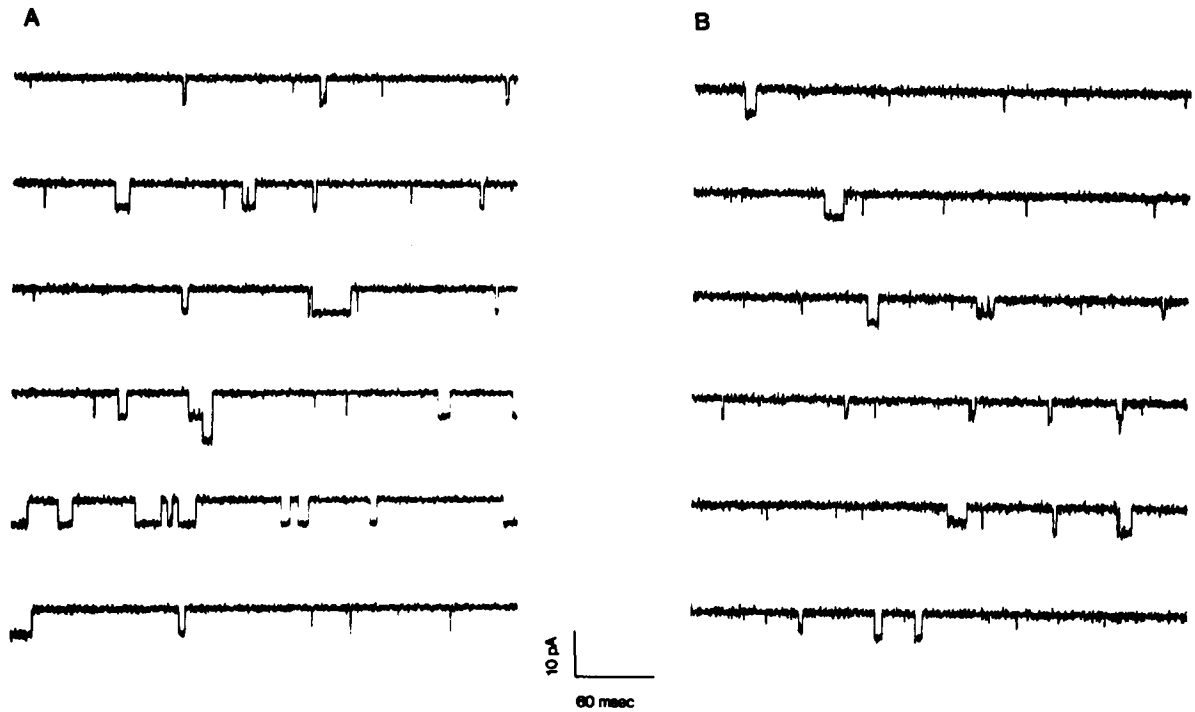

Fig. 1. Currents through AChR channel at a membrane potential of −90 mV, in cell attached patches for: (A) normal myocyte with a c/p molar ratio of 0.27; only one current level is observed. (B) A cholesterol enriched myocyte with a c/p molar ratio of 0.51; two single current level are clearly observed. Both myocytes were 9 days in culture.

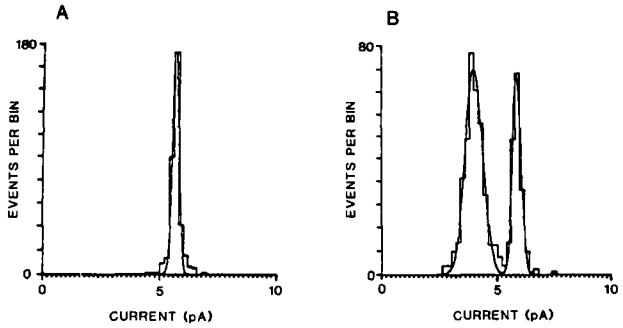

Fig. 2. Current amplitude histograms at holding potential of −90 mV in cell attached patches for: (A) a normal myocyte with a current of 5.66 pA, and (B) a cholesterol enriched myocyte which displays two major current peaks of 5.86 pA and a 3.99 pA. The data from these histograms belong to the patches illustrated in Fig. 1.

addition, the modulation time was detected and the apparent lifetimes by the phase-shift (τ_p) were smaller than the ones obtained by the demodulation factor (τ_m) (Table 2).

The patch-clamp experiments and the fluorescence measurements were performed under identical conditions, which means that the physical state of the plasma membrane of the myocytes was the same in both types of experiments. In addition, the patch clamp data was taken the same day in culture (between day 4 and 10) for both normal and enriched myocytes. Fig. 1 illustrates the main experimental observation and shows currents through ACh channels (0.10 μM ACh) in cell-attached patches for a control myocyte (9 day) (A) and in a cholesterol-enriched myocyte (9 day) (B) at a membrane potential of −90 mV. As shown, the control myocyte has only one current amplitude while the cholesterol-enriched myocyte has two. One of the amplitudes in (B) is significantly smaller than the control. In addition, the low amplitude channel appears to have a shorter open time and the noise level is higher. The other current amplitude in (B) seems to be very similar to the control, with a similar open time and noise level. In the absence of ACh, no current was observed in both types of myocytes. In order to verify that both current amplitudes are associated with nicotinic ACh-channel, con-

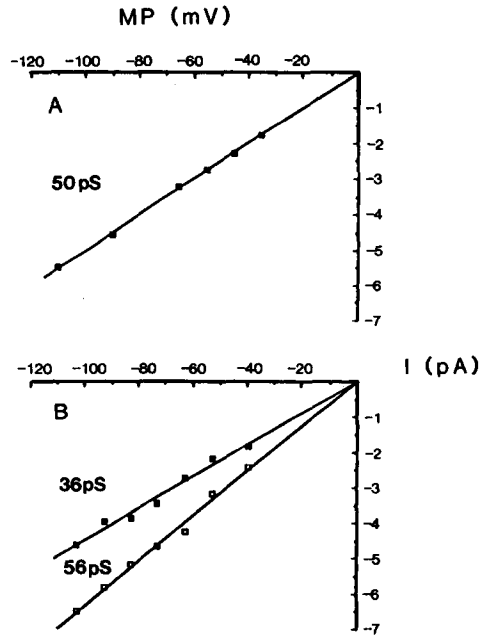

Fig. 3. Current–voltage relationships for: (A) a normal myocyte with a 50 pS conductance and (B) a cholesterol enriched myocyte with 56 pS and 36 pS conductances. The data were taken from cell-attached patches. Five independent patches with less than 6% difference in resting membrane potentials were used to estimate the average conductances of 54 pS and 39 pS.

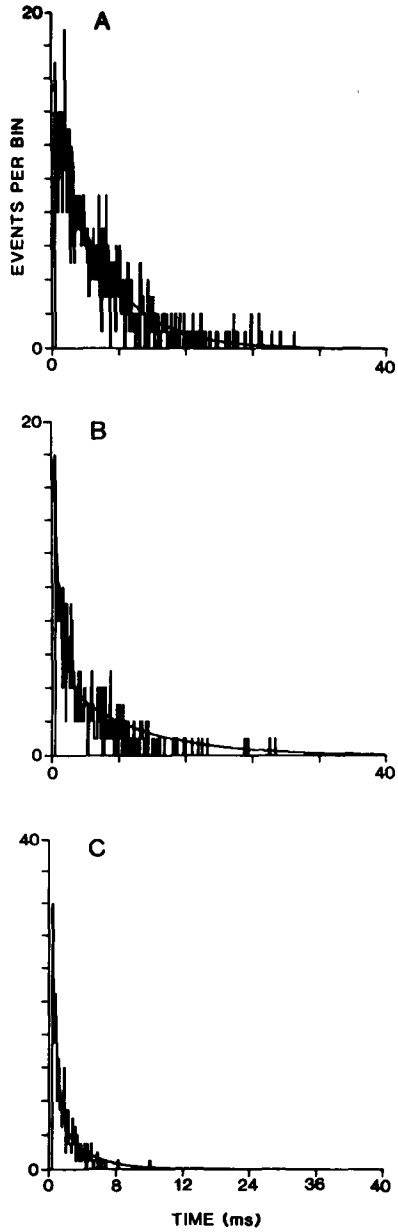

Fig. 4. Open time histograms in cell attached patches at −90 mV for (A) the 51 pS (control myocyte, 350 events) and (B) 54 pS (high conductance, cholesterol enriched, 352 events) and (C) 39 pS (low conductance, cholesterol enriched, 314 events). The 51 pS and 54 pS channel have very similar open time distributions. The 39 pS channel shows a very dramatic decrease (80%) in the open time constant relative to the normal channel.

trol experiments were performed in five batches of cholesterol enriched myocytes pre-incubated with 1 μM BuTx for 40 min. No channel activity was detected at membrane potentials between -40 and -140 mV. The current amplitude distributions for the recordings in Fig. 1 are shown in Fig. 2. At a membrane potential of -90 mV the control myocyte (A) has a current peak of 5.66 pA while the cholesterol enriched myocyte displays two well defined currents of 5.86 and 3.99 pA. The effect of cholesterol enrichment on the AChR conductance is shown in the I–V relationships in Fig. 3. The control myocyte (A) has a 50 pS conductance while the cholesterol-enriched myocytes display two conductances, 56 pS and 36 pS. From five patches, each from a different cell, the following values were obtained: 56 and 36 pS (Fig. 3), 54 and 38 pS, 55 and 42 pS, 59 and 40 pS and 57 and 37 pS. The average values for the cholesterol enriched myocyte were 39 ± 3 and 54 ± 2 pS. The average difference in the extrapolated reversal potentials between both conductances was not higher than 6.0 mV. It seems that the high conductance (54 pS) channel in the cholesterol enriched myocyte is very similar to the control since the difference in conductance was only 8%.

A very significant difference in open time between the 54 and 39 pS channels was observed and this is shown in Fig. 4. The open time histograms for the control (A), the 54 pS (B) and the 39 pS (C) at -90 mV were fitted using the sum of two exponentials. Values for the apparent open time constants were τ_1 1.58 ± 0.33 ms, τ_2 17.88 ± 2.92 ms for the control (A), τ_1 0.43 ± 0.08 ms, τ_2 16.95 ± 2.57 ms for the 54 pS and τ_1 0.59 ± 0.06 ms, τ_2 3.63 ± 1.09 ms for the 39 pS (means $\pm$ S.D., $n = 5$ for each one). Again, the 54 pS channel seems to be very similar to the control since the ratio for τ_2 between normal and enriched is 0.95. On the other hand, the τ_2 ratio for the 39 pS channel was 0.20. This means that the low conductance channel induced by cholesterol enrichment of these myocytes has a shorter open time by a factor of about 5 relative to the control channel.

4. Discussion

The above data suggest that after the cholesterol enrichment in the chick myocyte, the ACh receptors present in the cell membrane display heterogeneous properties. Its seems that the 54 pS channel is similar to the normal (51 pS) channel with a very small degree of perturbation. A new channel conductance (39 pS), reflects the major possible membrane perturbation as a consequence of the cholesterol enrichment. In terms of magnitude, the reduction in channel conductance for this 39 pS was about 24% compared to the control (51 pS) and an 80% reduction in open time constant (τ_2) was even more dramatic. The fact that the 39 pS channel was sensitive to α-BuTx and that it was not detected in the absence of ACh demonstrates that this is a nicotinic ACh-mediated channel. After the cholesterol enrichment, there was an increase of the microviscosity of the plasma membrane of these myocytes, which is consistent with the cholesterol to phospholipid molar ratios estimated from the plasma membrane fractions.

Although a comprehensive understanding of the manner in which cholesterol interacts with the lipid bilayer as well as with integral membrane proteins remain to be elucidated, some details of these interactions have become clearer during recent years. A variety of physical techniques have been used to establish that cholesterol reduces the relative number of gauche conformers in the liquid crystalline state [19,20] resulting in a significant ordering of the lipid hydrocarbon chains. This 'ordering effect' has been associated with the reduction in mean area per phospholipid or 'condensing effect' [21–23] and also to an inhibition of water penetration into the bilayer [24,25]. One of the techniques that has provided important clues about the influence of cholesterol on dynamic properties of artificial bilayers is the analysis of fluorescence anisotropy decays [26]. Among the probes used for this technique DPH has been one of the most popular and best characterized. In bilayers with mixed phospholipid species DPH can display a range of excited decay states and the addition of cholesterol reduces the range of decays [27]. In the presence of cholesterol the DPH orientational distributions are shifted in favor of an alignment parallel to the acyl side chains leading to an increase in the lifetime and a decrease in the range of decays [26]. The dehydration of the membrane bilayer induced by addition of cholesterol reduce water–probe interactions. Probe–water interactions result in dynamic quenching of fluorescence, leading to a decrease in the fluorophore lifetime. So far, our results with the chick myocytes are consistent with the data in artificial bilayers. The modulation time in the normal myocyte was probably too fast to be detected due to the presence of water and to less ordering in the membrane. Measurements of short lifetimes by the demodulation method has some degree of difficulty [28]. However, at the same modulation frequency the enriched myocytes had a well defined lifetime decay. If we assume that the dynamic fluorescence properties of DPH reflect global properties of the bilayer then the appearance of the τ_m in the enriched myocytes suggests an alteration in the bilayer properties by the inclusion of cholesterol.

In a membrane with high cholesterol levels, one could expect an inhibition of any protein, such as the AChR-channel, that needs to recruit sufficient volume elements for its conformational change. As an example, cholesterol was shown to modulate the conformational states of rhodopsin [29]. It is clear that two different conductances are present after the enrichment. This suggest that the AChR channel in the cholesterol enriched myocyte has two different types of conformational states. The 54 pS channel represents a similar conformational state relative to the 51 pS channel in the normal membrane and the 39

pS clearly represents an inhibited state. An attractive explanation for this result may be taken from the knowledge that cholesterol does not distribute homogeneously in cell membranes (for review see [30]). The simultaneous appearance of a longer τ_m with the 39 pS channel suggests the possibility that they are directly related. A very attractive hypothesis is that if τ_m reflects a rich cholesterol containing domain due to its longer lifetime, then the 39 pS channel should be located in a restricted motion microdomain. This seems to be a reasonable assumption since in this case it represents the inhibited conformational state. However, cholesterol has also been proposed to induce phospholipid segregation. This effect has been ascribed to the preferential interaction of cholesterol with saturated PC. A cholesterol enrichment in microsomal membranes has been shown to increase the fluidity of the protein-immobilized lipids [31]. The proposed hypothesis for that particular case was based on phosphatidylcholine (PC) segregation, where the saturated PC species supposedly segregate into the cholesterol rich sites and the unsaturated PC displaced toward the protein vicinity. From previous studies on the reconstituted *Torpedo* AChR, is well known that an optimal fluidity is necessary for ion channel flux [3]. Therefore, any of these two hypotheses might represent a possible mechanism. Another hypothesis that has support from previous work is a direct interaction of the sterol with the AChR. The observed inhibition also can be correlated with the possible interaction of the sterol molecule with specific sites that modulate the AChR function. These 'sterol sites' first proposed by McNamee's group [4] have also been proposed based on theoretical model of an α-helical structure on the M1, M3 and M4 transmembrane domains of the AChR [32]. In addition, other integral membrane proteins have been shown to interact directly with cholesterol [33,34].

An observation that deserves some comments is the attenuation of the 39 pS channel during first 4 days in culture (data not shown). This observation might be very important for the purpose of comparing different studies in which a lipid enrichment is performed on cultured cells. For example, in *Xenopus* myocytes, which contain two AChR conductances (44 and 67 pS), the addition of cholesterol did not produce any significant effect on the channel conductance [7]. However, the study did not mention in which day in culture the sterol enrichment was performed and the current amplitude histogram shown for the enriched cells actually seems to be different from the normal myocyte. One hypothesis to explain the attenuation of the appearance of the inhibited conductance (39 pS) in the early days in culture could be based on a previously proposed tight cholesterol–AChR interaction [6]. In the first 4 days in culture the high density of AChRs in the surface membrane of these cells might possibly sequester more cholesterol. This perhaps leads to a decrease in the degree of the cholesterol segregation in the bilayer. In order to find a possible answer to these hypotheses further experiments will concentrate on extracting rate constants for each conductance using burst analysis at high ACh concentrations, cholesterol depletion experiments and anesthetic action.

In summary, these results can be taken as additional evidence of lipid mediated alteration of integral membrane protein activity. We were able to show that a lipid alteration of the cell membrane may be reflected at the single channel level. In a single patch we were able to detect one type of AChR channel with two dramatic differences in physical properties, which was caused by an alteration of the cell membrane induced by high cholesterol levels. Learning how lipid components affects bilayer structure and dynamics in conjunction with functional information about integral membrane proteins would help to understand the functional nature of the lipid–protein interactions.

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