Biochemical and Biophysical Research Communications 505 (2018) 1038–1042

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Spin label EPR suggests the presence of cholesterol rich domains in cultured insect cell membranes

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ARTICLE INFO

Article history:
Received 24 September 2018
Accepted 2 October 2018
Available online 9 October 2018

Keywords:
Lipid rafts
Membrane nanodomains
Cholestane spin label
Membrane fluidity
Cholesterol
Cultured insect cells

ABSTRACT

Different spin labels were incorporated to the membranes of cultured insect UFL-AG-286 cells in order to characterize their physical properties by Electron Paramagnetic Resonance spectroscopy (EPR). The spectrum of the spin label 12-SASL incorporated to cell membranes was similar as those obtained in membrane model systems composed of eggPC/cholesterol. However, the spectrum of the spin label CSL, chemically related to cholesterol, was drastically different in the two systems. Interestingly, when cell cholesterol content was reduced using methyl beta cyclodextrin, an EPR spectrum similar to those of model membranes was obtained. The analysis of these experiments suggests the existence of cholesterol rich regions in UFL-AG-286 cell membranes.

1. Introduction

Cholesterol is an essential component of all animal cells, where it contributes to the modulation of membrane fluidity and rigidity [1,2]. Cholesterol is not homogeneously distributed inside the membrane of animal cells. Rather, it is concentrated into nanodomains known as lipid rafts or membrane rafts [3]. Lipid rafts are involved in signal transduction and materia exchange across membranes [4,5], including also the internalization of viruses [6,7].

Despite its essentiality, it has been described that the cells of insects contain a significantly lower proportion of cholesterol than mammalian cells [8]. Besides, several lepidopteran insect cell lines were adapted to culture media free of cholesterol, where they were cultivated almost indefinitely [9,10]. The presence of cholesterol-enriched microdomains has been described in membranes isolated from lepidopteran insects [11], but the existing knowledge about the order and dynamics of these microdomains in insect membranes is considerably scarcer than what is known about the characteristics of lipid rafts in the membranes of mammalian cells. Since microdomains enriched in cholesterol have been implicated in the interaction of insects with entomotoxins [11] and entomopathogenic viruses [12], deepening knowledge about the nature of lipid rafts in insect cells may have important implications in agriculture and biotechnology.

Mammalian cell lines have been a convenient model for studying cholesterol-enriched domains in mammals [13]. In the same way, insect cell lines can be useful to study their specific characteristics in insects. The cell line UFL-AG-286 [14] has several attractive and advantages to be used for this purpose. It has been established from embryonic tissues of Anticarsia gemmatalis, one of the main pests of soybean crops throughout the American continent, and is the cell line of choice for the in vitro propagation of the Anticarsia gemmatalis multiple nucleopolyhedrovirus, the baculovirus used as a biological control agent for agricultural plagues on a larger scale worldwide [15,16], besides to be a cellular substrate for the expression of recombinant products [17]. This cell line has been adapted to the culture in a serum-free medium, whose composition has been published and can be manipulated for experimental purposes [18].

The goal of the present work was to study the effects of cholesterol on order and dynamics of UFL-AG-286 cell membrane lipids. To this aim, EPR spectroscopy with two different kinds of membrane labels has been used, sensing different environments of the lipid bilayer. Stearic acid (n-SASL) and cholestane (CSL) spin

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https://doi.org/10.1016/j.bbrc.2018.10.011
0006-291X/

labels have molecular structures similar to those of membrane lipids, and bear a stable nitroxide radical. When incorporated in low proportions to model or natural membrane systems, they cause negligible perturbations in the system under study, and their EPR spectrum is sensitive to the dynamics of their micro environment, reflecting local sterical restrictions [19,20]. In this work, they were used to study the influence of cholesterol on lipid ordering in the plasma membrane of intact and cholesterol depleted UFL-AG-286 cells, and in egg- PC/Cholesterol large unilamellar liposomes (LUV) as model systems.

2. Materials and methods

2.1. Cell line

The UFL-AG-286 cell line [14] was kindly provided by Dr. Victor Romanowski (IBBM, National University of La Plata-CONICET, Argentina).

2.2. Cell cultures and culture medium

Adherent cultures of UFL-AG-286 cells in 25 cm^2 T-flasks (GBO, Germany) were performed in a home-made low-cost medium free of serum, optimized to cultivate this cell line, which detailed composition was published previously elsewhere [21]. The medium was used either as a basal medium without supplements, or supplemented with lipid microemulsions.

2.3. Lipid emulsions

The microemulsion utilized for cholesterol supply was prepared as follows. Soy oil (0.1 g), Tween 80 (0.25 g), cholesterol (0.045 g) and α-tocopherol (0.02 g) were weighted and diluted in 10 ml of absolute ethanol. One ml of this ethanolic solution was deposited into a 50 ml tube, and 10 ml of Pluronic F68 10% in water were added drop by drop, with vortex agitation. The resulting microemulsion was dissolved in the basal culture medium at 1% concentration. A modified microemulsion omitting cholesterol and α-tocopherol was prepared to dissolve the spin label CSL.

2.4. Lipids and spin labels

Pure lipids were purchased from Sigma Chemical Co. (St. Louis, USA) and Avanti Polar Lipids (Alabaster, USA). The liposoluble spin labels 5-, 12-, 16-SASL and CSL (cholestane spin label) were from Sigma. The molecular structures of these labels are shown in Fig. S1 of the Supplementary Material. Solvents, inorganic salts and all other chemicals were of the highest available purity.

2.5. Preparation of spin labeled liposomes as membrane model systems

Large unilamellar vesicles (LUV) were prepared with egg phosphatidylcholine (egg-PC) with different nominal cholesterol contents: 0, 4, 10, 20, and 40 mol% relative to total lipids, and 1 mol% of one of the spin labels. In brief, the dry lipid mixture of each composition was dissolved in chloroform, the adequate amount of a stock solution of the corresponding spin label was added, and the organic solution was spread on the bottom of a rounded glass tube. The solvent was evaporated with a N_2 stream, and the tube was left under vacuum overnight. Next, the dry lipid film was hydrated with PBS (phosphate buffer saline, pH 7.3) at 40 °C with vortex agitation to a final lipid concentration of 18.5 mM. The resulting multilamellar liposome solution was extruded through 100 nm pore filters at 40 °C with an Avanti Mini-Extruder. As the actual LUV cholesterol content was not measured, small deviations from the nominal egg-PC/cholesterol values could be expected, which are not relevant for the purposes of our work.

2.6. Cholesterol depletion of UFL-AG-286 cells

Cells collected from stationary cultures of UFL-AG-286 cells (1×10^7 viable cells) were washed three times with PBS and incubated in methyl beta cyclodextrin (MβCD) 15 mM, 5%V/V, by one hour at 27 °C on an orbital shaker at 100 rpm. Subsequently cells were washed with basal culture medium free of cholesterol and centrifuged at low speed (168 ×*g*) during 3 min. Cell viability was quantified by the Trypan blue method.

2.7. Spin labeling of UFL-AG-286 cells

In all cases the amount of spin label was calculated in order to obtain a final concentration of about 1% relative to the total membrane lipids. The procedure of labeling differed between the n-SASL and CSL, because the last label is insoluble in water.

2.7.1. Spin labels n-SASL

The calculated amount of ethanol stock solution of 5-, 12- or 16-SASL (0.2 μl) was deposited in an eppendorf tube, and after the solvent evaporation, 1×10^7 cells suspended in basal culture medium (without emulsion) were added. The mixture was homogenized and immediately centrifuged and washed once in basal culture medium. Centrifugation steps were at 168 ×*g* for 3 min. The packed cells were introduced in a capillary tube which was flame sealed, and the EPR spectra were acquired within 15 min of the initial contact of the cells with the spin labels.

2.7.2. Spin label CSL

This label, analogue of cholesterol, is insoluble in aqueous media. The stock solutions are prepared in chloroform, which is toxic to cells. Two μl of CSL stock solution were deposited into a glass tube, and the solvent was completely evaporated by flushing with dry N_2. In order to solubilize the deposited CSL, 11 μl of the modified emulsion were added with vortexing. Then, 1 ml of 1×10^7 cells in basal medium was added, incubated at 27 °C for 10 min and afterwards centrifuged at 168 ×*g*, 3 min. The supernatant was reserved in order to quantify the amount of CSL not incorporated by the cells. The pellet was washed with basal medium and centrifuged, reserving also the supernatant. The packed cells were put into glass capillaries and immediately measured by EPR.

In order to assess the amount of CSL effectively incorporated to UFL-AG-286 cells, the incubation supernatant, the washing supernatant, and the remaining cells adhered to the incubation tube were each extracted with 100 μl of chloroform. Fifteen μl of each of the samples were loaded into glass capillaries, and EPR spectra were obtained. Their intensities were compared to that of a standard solution of CSL in chloroform.

2.8. EPR experiments and EPR data analysis

EPR spectra were acquired with a Bruker EMX-Plus spectrometer (Bruker Analytische Messtechnik GMbH, Karlsruhe, Germany), at (27 ± 1) °C. Microwave frequency was centered in 9.8 GHz (X band), modulation frequency was 100 kHz and microwave power was 3 mW.

2.8.1. Spectra of the spin labels n-SASL

Due to the different positions of the nitroxide moiety in the stearic acid chain (Fig. S1), these labels sense the mobility of hydrocarbon lipid chains at different depths: near the lipid polar

heads (5-SASL), in the intermediate region of the hemilayer (12-SASL), and in the middle of the lipid bilayer (16-SASL). As an example, Fig. S2 shows the EPR spectra of 5-, 12- and 16-SASL in pure egg-PC liposomes. The observed spectral band structure is due to the resolved magnetic hyperfine interaction with the ^{14}N nucleus present in the nitroxide radical. Position and width of the EPR bands are sensitive to membrane fluidity and order, which explains the differences among the spectra at the three depths. A good parameter to estimate membrane fluidity is the hyperfine parameter A_{MIN}, calculated as the half difference in magnetic field values at the arrow positions shown in the insert of Fig. 1. This parameter increases with increasing fluidity and/or decreasing order.

2.8.2. Spectra of the spin label CSL

The spectra of CSL in LUV of egg-PC also show the three bands due to the resolved magnetic hyperfine interaction, but they have a different shape in comparison of those of n-SASL due to the different rotational axis of the nitroxide radical in CSL [22]. In these spectra, it is convenient to quantify fluidity with the hyperfine parameter A_{MAX}, calculated as half the difference in magnetic field values at the arrow positions shown in the insert of Fig. 3. It should be remarked that this parameter increases with decreasing fluidity/increasing order, opposite to the behavior of A_{MIN}.

For both types of spin labels, the necessary condition to observe undistorted EPR spectra is the adequate dilution of the labeled molecules, typically less than 2 mol% relative to total lipids. Increased concentrations would lead to gradual widening and shifting of the spectral bands, leading ultimately to a completely collapsed structure due to the magnetic exchange interaction in the case of high spin label concentration [23].

3. Results and discussion

3.1. Spin label 12-SASL in LUV

Fig. 1 shows A_{MIN} values obtained for 12-SASL in egg-PC/Chol LUV as a function of their relative nominal cholesterol content. A monotonous decrease in A_{MIN}, indicating a decrease in membrane fluidity, is observed up to the maximum cholesterol content of 40%. Consistently with our results, Gallová et al. reported increased lipid chain order in egg-PC liposomes with increasing cholesterol concentration, using 16-SASL [24]. It was observed that 12-SASL is the most sensitive of the n-SASL to cholesterol variations (Fig. S3, showing A_{MIN} for the three spin labels), indicating that fluidity changes at the region sensed by this label are the most important. This is probably due to the fact that the mean depth of the nitroxide moiety of this label is around carbons C9 and C10 of its lipid neighbors, sensing better the effects of cholesterol [25].

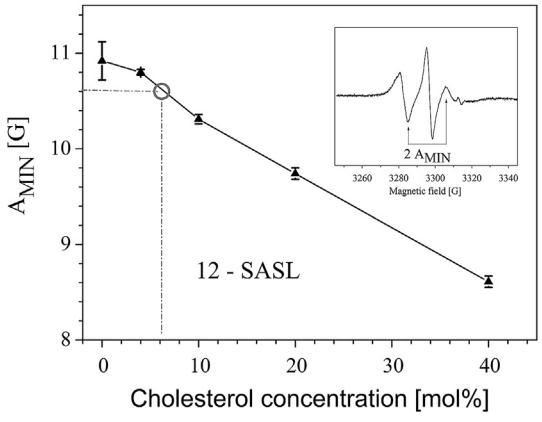

Fig. 1. Hyperfine parameter A_{MIN} of the stearic acid spin label 12-SASL in LUVs egg-PC/cholesterol with different nominal cholesterol contents. Hollow circle: A_{MIN} values measured in intact UFL-AG-286 cells. Temperature: (27 ± 1) °C. Insert: EPR spectrum of 12-SASL in egg-PC. The position of the peaks involved in the determination of A_{MIN} is identified with arrows.

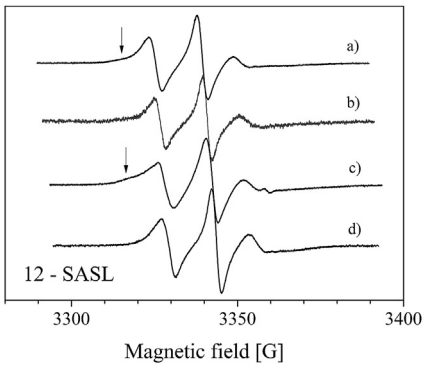

Fig. 2. EPR spectra of the spin label 12-SASL in UFL-AG-286 cells and in liposomes. (a) Intact cells; (b) cells treated with MβCD; (c) cells treated with MβCD and re-fed with cholesterol; (d) LUVs of egg-PC with nominal 10% cholesterol. The arrows point to the shoulder appearing in the case of cells with normal cholesterol content. Temperature: (27 ± 1) °C.

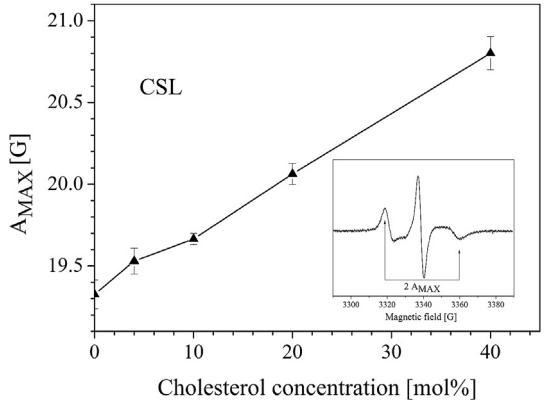

Fig. 3. Hyperfine parameter A_{MAX} of the spin label CSL in egg-PC/cholesterol LUVs with different nominal cholesterol contents. Insert: EPR spectrum of CSL in LUVs of egg-PC. The position of the peaks involved in the determination of the A_{MAX} parameter is identified by arrows. Temperature: (27 ± 1) °C. The increase in A_{MAX} indicates a decrease in membrane fluidity as cholesterol concentration increases.

3.2. Spin label 12-SASL in UFL-AG-286 cells

Intact and cholesterol depleted cells were labeled with 12-SASL. Similar spectra to those of 12-SASL in LUV were obtained. The mean value of the hyperfine parameter A_{MIN} was (10.60 ± 0.08) G in intact cells, and (11.00 ± 0.06) G in cholesterol depleted cells, consistent with an increased fluidity at decreased cholesterol levels.

In order to verify that UFL-AG-286 cells tend to preserve a characteristic fluidity regardless of their feeding history, one aliquot of intact cells was cholesterol depleted with MβCD, and subsequently re-fed with cholesterol microemulsion. Both intact and re-fed cells were labeled with 12-SASL, and the values of A_{MIN} obtained from their EPR spectra were (10.60 ± 0.08) G (intact cells), and (10.51 ± 0.08) G (re-fed cells). The coincidence between these two results indicates that UFL-AG-286 cells tend to reach a stationary status having a fixed amount of membrane cholesterol, independently of their feeding history.

The values of A_{MIN} obtained in cell samples can be compared

with those measured in LUV (Fig. 1). It can be observed that A_{MIN} values of intact and re-fed cells would correspond to A_{MIN} in LUV with around 6% nominal cholesterol (see circle in Fig. 1), coincident with the reported cholesterol concentration in insect cells [8]. On the other hand, A_{MIN} for cholesterol depleted cells would correspond to that of LUV of PC without cholesterol.

Fig. 2 compares the EPR spectra of 12-SASL in the different UFL-AG-286 cell samples. A spectrum of 12-SASL in LUV of egg-PC/cholesterol is also added.

When comparing these spectra, a shoulder at low fields can be observed (pointed to by arrows in Fig. 2) for the case of intact cells and cholesterol depleted and re-fed cells. This shoulder is absent in the spectra of cholesterol depleted cells and LUVs. This fact indicates that in cells with normal cholesterol content, the spin label partitions in membrane regions of different fluidity, the shoulder corresponding to less fluid membrane portions. A similar behavior was reported by Swamy et al. for mammalian cell lines labeled with n-PC spin labels [26].

3.3. Spin label CSL in egg-PC/cholesterol liposomes (LUVs)

EPR spectra of CSL in LUV of egg-PC containing different nominal amounts of cholesterol up to 40% were acquired at 27 °C (the insert of Fig. 3 shows the spectrum of CSL in pure egg-PC). Fig. 3 shows the values of A_{MAX} as a function of the nominal cholesterol concentration. The increased A_{MAX} indicates a reduction in fluidity with cholesterol concentration, in coincidence with the results obtained with 12-SASL.

3.4. Spin label CSL in UFL-AG-286 cells

The EPR spectrum of CSL in intact cells is shown in Fig. 4a. This spectrum is drastically different from the previous ones, because it lacks the hyperfine structure, which is collapsed to a single, broad spectral band. The lineshape is nearly lorentzian, consistent with an exchange collapsed spectrum [23]. The dashed line is the fit to a lorentzian lineshape. The peak to peak linewidth is in this case

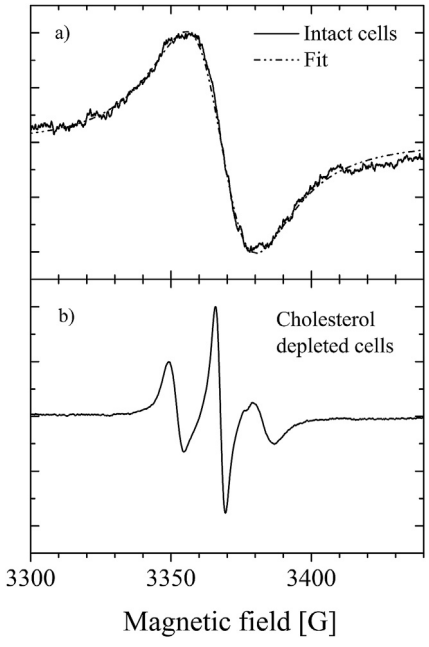

Fig. 4. EPR spectra of the spin label CSL in UFL-AG-286 cells. (a) Intact cells (full line), fitted with a lorentzian lineshape (dot-dashed line); (b) cholesterol depleted cells, at short times. Temperature: (27 ± 1) °C.

20.3 G, but it varies between 20 and 24 G for intact cells grown in different batches. The amplitude of this spectrum does not decrease in time.

In order to analyze the influence of cholesterol on the spectral collapse, cells were depleted in cholesterol with MβCD, and labeled with CSL. The resulting EPR spectrum, obtained 18 min after putting the spin label in contact with the cell suspension, is shown in Fig. 4b. In this case, a resolved three line spectrum is observed, similar to those obtained with CSL in LUVs. The hyperfine parameter A_{MAX} is (19.1 ± 0.3) G, which is coincident with that of LUV of pure egg-PC (Fig. 3). The intensity of this resolved spectrum decreases with time, with characteristic times varying between 5 and 35 min for the different samples. A typical time evolution is shown in Fig. S4. After a time long enough, a remaining collapsed spectrum was observed. A careful inspection revealed that this collapsed spectrum was already present in the first spectrum of the time evolution, in the form of a baseline. In fact, a subtraction of the collapsed long time spectrum from the resolved first time spectrum removes the observed baseline, indicating that the short time spectrum is a superposition of a hyperfine resolved, LUV-like spectrum, plus a collapsed, intact cell-type spectrum. Fig. S5 shows this phenomenon.

The necessary condition for observing an exchange collapsed EPR spectrum is the overlap of the wavefunctions of the unpaired electron spins of neighbor nitroxide radicals, yielding a high enough exchange frequency [23]. This implies a high concentration c_{CSL} of the spin label. However, the amount of CSL used in intact cells (about 1 mole% relative to the estimated amount of membrane lipids) is the same as in cholesterol depleted cells, where a resolved hyperfine spectrum is observed. Thus, we are forced to assume that the collapsed spectrum observed in intact cells is due to CSL segregated in certain membrane regions in which its local concentration c_{CSL} is much higher than the average. As CLS is a molecule chemically akin to cholesterol, it is expected that the membrane regions with higher c_{CSL} would be those enriched in cholesterol, i.e. membrane rafts [3]. Grammenos et al. also observed a collapsed EPR spectrum of CSL in human carcinoma cells [27]. The presence of clusters formed solely by CSL molecules is discarded, as the EPR spectrum of pure CSL samples has a linewidth of 15 G, smaller than in intact cells (Fig. S6).

The picture emerging from the EPR results is that the fate of CSL molecules is different when they are incorporated to intact and to cholesterol depleted UFL-AG-286 cells. In the first case, CSL molecules are recruited and segregated to regions of high cholesterol concentration, supposedly membrane rafts. These regions must be small enough to force the proximity of CSL molecules. A rough calculation based on the linewidth of the collapsed EPR spectrum allowed us to estimate a distance of 1.4 nm among neighbor CSL molecules located in rafts, contrasted to 4.6 nm calculated for a homogeneous distribution of 1% CSL in the cell membrane. As the intensity of the collapsed EPR spectrum remains constant, we can conclude that raft recruited CSL molecules cannot be reached by antioxidant molecules. In the case of cholesterol depleted cells, rafts are expected to be disrupted [28,29], and most of the incorporated CSL molecules would remain randomly distributed in the membrane, yielding a resolved EPR spectrum similar to that observed in LUVs. This spectrum decreases with time, possibly due to the action of membrane antioxidants. The collapsed spectral component appearing in these cells superimposed to the resolved one (Fig. S5) could be attributed to CSL in residual rafts survivors of cholesterol depletion.

In conclusion, we have performed an EPR study of cultured insect cells using two spin labels with different chemical affinities. The spin label CSL, chemically akin to cholesterol, showed a collapsed spectrum -indicative of a high local CSL concentration-in

intact cells, but not in cholesterol depleted ones. Rough estimations of CSL distances suggest the presence of cholesterol enriched nanodomains in intact cell membranes. As the spin label 12-SASL is not expected to have a chemical affinity with cholesterol, the fluidity values obtained in UFL-AG-286 cells should be considered to reflect the behavior of the phospholipid rich, fluid portions of the cell membrane. The shoulder in the spectrum of cells with normal cholesterol content could be attributed to the location of 12-SASL in the vicinity of cholesterol nanodomains. To our knowledge, this is the first time that spin label EPR is used to study the biophysical properties of cultured insect cell membranes.

The elucidation of the particular characteristics of cholesterol-enriched nanodomains in insect cell membranes could help to understand how insects interact with entomopathogenic viruses and toxins, driving to new strategies for biocontrol of insect plagues, as well as to improve biotechnological processes based in insect cells.

Acknowledgements

This work was supported by Universidad Nacional del Litoral (Grants CAI + D 50120110100007 and 5012015010011LI) and CONICET (Grant PIP 112-20110100895), Argentina. AMG is a researcher of CONICET.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.10.011.

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