Decay Dynamics of Nystatin Channels in the Presence of Sterol Microstructures on Membranes

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Abstract

Nystatin (nys) is an antifungal agent which preferentially forms ion channels in membranes containing the sterol ergosterol (erg). The structure of the nystatin channel is not well understood, although it is known that multiple nystatin monomers require a sterol-rich membrane for aggregation. When nys/erg containing vesicles are fused to a sterol-free membrane, characteristic spike changes in membrane conductance are observed. The decay of a conductance spike is generally step-wise linear and the time of decay is a strong function of the concentration of ergosterol. These data indicate interdependence among the nys/erg channels. A model has been developed in which nys channels form at the boundary of a lipid/erg superlattice domains (ESLD) and channel decay is determined by erg diffusion from the ESLD. This model was tested using Monte Carlo (MC) simulations for the diffusion current from the ESLD. The MC simulations predict a constant diffusion current from the ESLD which results in a rate of loss of channels which is linear in time. The steps in the decay are understood in terms of multiple ESLDs and ESLDs with fissures. The model then provides a complete understanding of the decay scheme observed experimentally. This interpretation also predicts previously confusing data relating conductance spike height to vesicle diameter.

Keywords: superlattice, Monte Carlo simulation, channels, membrane

1. Introduction

In the presence of ergosterol, nystatin forms ion channels in a cell membrane, which lead to cell death by disrupting the normal intracellular environment. Coutinho, Prieto and coworkers discovered that molecules composed of nystatin monomers partition into the membrane surface and reversibly assemble into aggregates of 4 - 12 antibiotic molecules1,2. This property enables nystatin to be used as topical antifungal treatment. Nystatin can also be used to detect fusion between a vesicle and a phospholipid bilayer. When a vesicle with nystatin/ergosterol channels in its membrane fuses with a planar phospholipid bilayer, the vesicle membrane along with the channels become part of the bilayer. The presence of these new ion channels produces a sudden spike in the electrical current across the bilayer if a potential is placed across it. Assuming the bilayer does not contain ergosterol, the current spike decays as a direct result of the ergosterol from the vesicle diffusing into the phospholipid bilayer and the resulting collapse of the nystatin/ergosterol channels.

Ergosterol superlattice domains (ESLDs) are 2-dimensional lipid configurations that are typically composed of significant numbers of sterols (e.g. cholesterol or ergosterol). A superlattice is a lattice within a lattice. A sterol superlattice is formed when the sterol molecules are arranged in a regular pattern with each sterol occupying an acyl chain site within a lattice of lipid acyl chains. Monte Carlo simulations performed by Huang and Feigenson have revealed that the interactions which must be considered as a part of any attempt to understand the formation of sterol superlatices are very complex3,4.
This investigation reported here deals with the dynamics of the decay of the nystatin-ergosterol ion channels in the membrane which result from the fusion of vesicles containing these channels with the membrane. In previous studies conducted at Goshen College, vesicles were prepared with ergosterol mol fractions which presumably led to the formation of superlattice structures on the vesicle membranes. It is almost certain therefore, that ergosterol superlattice domains, or at least boundaries between ordered and disordered regions, exist on the vesicle membrane before fusion. During the fusion the membrane of the vesicle is placed under extreme stress. While it is possible that the vesicle membrane has sufficient cohesive forces to remain intact, it is more likely that the vesicle membrane breaks up into the sort of circular regions identified by Baumgart, et al. Therefore, this study focuses on the decay of groups of channels in the presence ESLDs.

In this paper a model is proposed to explain the experimental evidence reported by Weaver and Oyeyemi which demonstrates that the nystatin-ergosterol channels are not independent of one another and that their decay is strongly dependent on the mol fraction of ergosterol present in the vesicles. This model was subjected to Monte Carlo simulation studies. It will be shown that this model successfully accounts for the experimental observations reported by Weaver and Oyeyemi.

2. The Model

In the proposed model it is assumed that the nystatin/ergosterol channels are located around the perimeter of the ESLDs and that the channels are tied to the ergosterol molecules, which compose the superlattices. This comes as a result of the regular structure of the comparatively rigid ESLD superlattice, which denies other molecules easy access. It is expected that ESLDs which possess the same composition of ergosterol as the vesicle membrane are present in the small region of the bilayer where the fusion has occurred. Weaver and Oyeyemi have shown that nystatin-ergosterol channels are interdependent implies that the channels are thus functionally related to the ESLDs.

Neither the precise form of the nystatin/sterol nor the nystatin/lipid interactions are known. These interactions, however, determine the linear density of the nystatin/ergosterol channels (channels per unit length) around the circumference of the superlattice. As a consequence any change in the circumference of the superlattice region will result in a corresponding change in the number of channels. A conceptual depiction of the model is presented in Figure 1. While it represents an accurate illustration of the general organization of ESLDs, the size of actual ESLDs on a vesicle is likely much larger than shown in Figure 1.

The diffusion of the ergosterol from the superlattices into the bilayer correspondingly decreases the size of the ESLD. Since, for any given bilayer potential, the bilayer current is proportional to the number of channels, a decrease in ESLD circumference will result in a decay of the bilayer current. Thus the decay in bilayer current is a result of the diffusion of ergosterols away from the ESLD.

Monte Carlo studies of diffusion of ergosterols from the superlattice described below showed that the diffusion current of ergosterols out from the ESLD is best treated as a consequence of the direct release of the ergosterol from the superlattice structure. Thus only the ergosterols in the superlattice are treated.
3. Method

Monte Carlo simulations were conducted in order to investigate the microscopic details of the diffusion of sterols from an ESLD. The Monte Carlo approach is based on a time independent solution to a Markovian master equation in which the transition rates are chosen so that the simulation seeks a molecular configuration which is consistent with a Gibbs/Boltzmann distribution of the system energies\(^9\). As such, this approach is fundamentally statistical in nature and not exact physics. It is, however, consistent with the assumption that local thermodynamic equilibrium is reached on the time scales which are of interest.

Each Monte Carlo step entails a statistically selected move to a neighboring lattice site for each sterol molecule in the system being considered (the ESLD). The acceptance of each selected move is also statistical, but biased such that accepted changes in molecular configuration result in the movement of the entire system toward a local Gibbs/Boltzmann thermodynamic equilibrium. Thus a Monte Carlo step is a step in an algorithm and is not necessarily a fixed time step.

In the Monte Carlo simulations which were performed, a Metropolis algorithm and standard helical boundary conditions for the hexagonal lattice of lipid acyl chains were used. The Hamiltonian which was employed was that of Huang and Feigenson for which their set of energy parameters MIEP IV was used with a strength of the sterol multibody interaction equal to \(3kT\)^3,4. The computer code was written in Maple 9.5 and was run on a PC with a 1.5 GHz Athlon microprocessor.

As a test of the routine and its capabilities, the results of the Monte Carlo simulations for the superlattices presented by Huang and Feigenson were reproduced. The initial condition for the simulations was that all lattice sites in one region of the lattice were filled with sterols. The result for a 0.50 mol fraction of ergosterol (\(\chi_{erg} = 0.50\)) and 5600 Monte Carlo steps was identical to Huang and Feigenson’s Figure 2 (b) under comparable conditions\(^3\).

Because of the inherent mathematical difficulties of attempting to deal with a circular ESLD, this study was limited to determining diffusion currents from lines of symmetry within the superlattice. These linear sections can be used to approximate the perimeter of a circle. In Figure 2, a portion of the perimeter of a circular ESLD with \(\chi_{erg} = 0.50\) and symmetry lines has been constructed.

![Figure 2. Reconstruction of ESLD perimeter using symmetry lines for \(\chi_{erg} = 0.50\). The grey arrows indicate current densities perpendicular to the symmetry lines.](image)

This construction provides a reasonable approximation to a circular ESLD for the purposes of studying the diffusion current density from the ESLD. The net current from the ESLD is obtained from the product of the current densities in each direction and the segments of the ESLD perimeter which are perpendicular to each current density. The current densities corresponding to the gray arrows (A) and (C) are perpendicular to lines along which the ergosterol densities are equal. This is also true of the gray arrows (B) and (D).

In each of the primary directions indicated by the gray arrows, a two dimensional problem was considered. As initial conditions for the Monte Carlo simulation, a group of mobile sterols was arranged to be studied in an appropriate superlattice. One boundary was provided by a set of fixed sterols arranged in a superlattice, while the other boundary was provided by a region of pure lipid. The initial condition for the directions (B) and (D) and \(\chi_{erg} = 0.50\) is shown in Figure 3 (a). Figures 3(b) and 3(c) show the diffusion current emerging from the boundary of the superlattice (the perimeter of the ESLD) after 15 and 70 Monte Carlo steps respectively. For each frame there is a region to the left, not shown, that contains a fixed superlattice. Only the sterols which are free to move during the course of the simulation are shown. The number of these sterols was included as a parameter in the program.
4. Results

It was found that after a few Monte Carlo steps a steady state condition was reached in the region immediately beyond the boundary of the superlattice for each direction of symmetry. This corresponded to a steady diffusion current out from the superlattice in all directions. A plot of the number of sterols freed from a superlattice with $\chi_{\text{erg}} = 0.40$ as a function of the number of Monte Carlo steps is presented in Figure 4.

The straight line in Figure 4 corresponds to a least mean square fit of the data beyond Monte Carlo step 15. The linear character of this fit indicates a constant diffusion current if the Monte Carlo step is considered as a measure of the time as argued by Weaver and Oyeyemi\textsuperscript{7}. Because there is currently no way by which to determine the condition at the ESLD boundary immediately subsequent to vesicle fusion, the initial Monte Carlo steps possess no physical meaning and are simply required for establishing this steady current. Thus these initial points were not used when the data was fitted.
The Monte Carlo simulations thus demonstrated that the diffusion flux of the ergosterol molecules was essentially constant around the perimeter of the ESLD (the diffusion current was constant). Specifically it was found that the ratios of the radial diffusion current densities from the directions (D) and (C) of Fig. 3 were $1.10 \pm 0.28$ at $\chi_{\text{erg}} = 0.40$ and $1.11 \pm 0.27$ at $\chi_{\text{erg}} = 0.50$. Slight differences in the diffusion current densities in the different directions were expected as a result of the fact that the energy change of removing a sterol from the superlattice is affected by the immediate environment of the particular sterol. This corresponds to a macroscopic, thermodynamic picture in which diffusion is established by a gradient in the chemical potential which is uniform around the perimeter of a circular ESLD.

In Appendix A, a relationship is developed between the diffusion current flux from the ESLD and the rate of decay of the membrane current due to nystatin ergosterol channels.

5. Discussion

This paper was a theoretical test of the model proposed to understand the basis of the experimental results presented by Weaver and Oyeyemi. The principal elements of the model are: the decay of channels is a result of the decay of ESLDs, the decay of an ESLD results from diffusion of sterols away from the superlattice into the region of pure lipid, the ESLDs are generally circular, and the channels are tied to the ESLDs along the perimeter. The theoretical investigation of the model took the form of Monte Carlo simulation. Weaver and Oyeyemi experimentally observe a marked difference in the current spike duration and provide a justification for this in terms of molecular interactions.

The Monte Carlo simulations have shown that the diffusion flux of ergosterol from an ESLD is constant. The relationship developed in Appendix A then predicts that the rate of membrane current decay from an ESLD is constant and, therefore, that the membrane current decreases as a linear function of the time. The model then accounts for the linear decay schemes identified by Weaver and Oyeyemi and presented in Figure 6.

The model does, however, make no statement with regard to the molecular interactions which occur between the nystatin channels and either the sterols of the superlattice or those diffusing away from it. Specifically, the question of the dynamics of channel separation from an ESLD is not considered by the model. The model requires solely that separation from the ESLD results in the loss of the channel as a functional unit.

The proposed model is also required to account for the multiple slopes within the fusion spikes which are often observed. As noted in the Introduction, vesicle fusion may produce a number of ESLDs with each one exhibiting the same superlattice structure as the original vesicle. The stresses experienced by the vesicle in the act of fusion are likely to produce more complex configurations than that of the single superlattice shown in Figure 1. Figure 5 depicts such a configuration, which was adapted from several different frames of the superlattice development that resulted from the Monte Carlo simulations. The ESLD contains a fissure and symbolic channels along both the external and internal superlattice boundaries.

![Figure 5](image)

Figure 5. An ESLD with a fissure and channels. The symbols have the same meaning as in Figure 1 and outwardly diffusing ergosterol molecules have been added manually.

As a specific example, consider the case in which two ESLDs are formed in the membrane after vesicle fusion has taken place. The first ESLD has a uniform interior, as in Figure 1, but is much larger than depicted. The second ESLD is even larger and, as in Figure 5, possesses an irregular internal fissure. The kinetics of the nystatin channel decay would be expected to mirror the observed bilayer current decay patterns observed by Weaver and Oyeyemi and presented in Figure 6. The steep initial decay of the current can be explained by the loss of channels from the
perimeters of both ESLDs which occur as ergosterol diffuses out of the ESLD. The smaller, uniform ESLD disappears completely before the second ESLD and its departure is marked by a decrease in the current slope. The second ESLD continues to shrink until the point where the perimeter intersects the boundary of the internal fissure. At this point, the second ESLD has effectively split and formed two separate ESLDs. This event is accompanied by a corresponding increase in the current slope which will then be followed by another decrease when one of the new ESLDs disappears. Once all of the ESLDs have disappeared and all the channels are gone, the bilayer will again return to baseline, as seen in Figure 6 and observed by Weaver and Oyeyemi. Thus, the model can be applied to a relatively simple combination of ESLDs bordered with nystatin channels in order to predict bilayer current decays which are identical to those observed experimentally.

Figure 6. Bilayer current vs. time for $\chi_{\text{erg}} = 0.245$. Experimental data from Weaver and Oyeyemi.

The conclusion that nystatin channels form at lipid domain boundaries predicts a linear relationship between the size of a vesicle and the number of channels in a vesicle. If, in contrast, nystatin channels were to form within a superlattice, or throughout the entire vesicle, the number of channels would then be proportional to the surface area of the vesicle and thus to the square of the vesicle diameter. It has been shown previously that channel number is not proportional to the square of the diameter, but is linear or sublinear and thus proportional to the diameter. This is demonstrated in Figure 7.

Figure 7. Average fusion spike current is a linear function of vesicle diameter not of vesicle surface area.

An analysis presented by Weaver and Oyeyemi shows that the proposed model also accounts well for a dramatic difference in spike duration between $\chi_{\text{erg}} = 0.40$ and $0.50$. Upon detailed analysis the proposed model has then accounted for all of the present as well as previously obtained (and unexplained) experimental results. It is believed, therefore, that it can be used as a basis for further study of nystatin/sterol channels including the form of the chemical linkage between nystatin and the sterol discussed above.

6. Appendix A

Consider a circular superlattice of radius $r$ with $\sigma_S$ ergosterols per unit area and a total of $N_S$ ergosterols. Let there be $n_C$ channels per unit length around the circumference of the superlattice with each channel occupying a length $\lambda_C$ of the circumference. Each channel carries a current $I_C$. The rate of loss of ergosterols from the superlattice is
where $A$ is a constant, $\delta \mu$ is the difference between the chemical potential of the superlattice and the immediate surroundings and $T$ is the absolute temperature. The area of the superlattice is $\pi r^2 = N_S / \sigma_S$. Then

$$\frac{dN_S}{dt} = \frac{2\pi Ar}{T} \left(1 - n_c \lambda_c\right) \delta \mu$$
(A.1)

The number of channels on a raft of radius $r$ is $N_C = 2\pi n_C$. Then

$$2\pi \frac{dr}{dt} = \frac{1}{\sigma_S} \frac{2\pi Ar}{T} \left(1 - n_c \lambda_c\right) \delta \mu.$$  
(A.2)

Each channel is assumed to carry a current $I_C$ until the channel becomes detached from the superlattice and is no longer functional. The rate of change of the bilayer current is then

$$\frac{dI_{ESLD}}{dt} = 2\pi n_C \frac{A}{\sigma_S} \left(1 - n_c \lambda_c\right) \delta \mu$$  
(A.4)

which is a constant.

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8. References