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The role of sterols in the lipid vesicle response induced by the pore-forming agent nystatin

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ABSTRACT

The influences of ergosterol and cholesterol on the activity of the nystatin membrane were investigated experimentally in a POPC model membrane as well as theoretically. The behavior of giant unilamellar vesicles (GUVs) under osmotic stress due to the formation of transmembrane pores was observed on single vesicles at different nystatin concentrations using phase-contrast microscopy. A significant shift of the typical vesicle behavior, i.e., morphological alterations, membrane bursts, slow vesicle ruptures and explosions, towards lower nystatin concentrations was detected in the ergosterol-containing vesicles and a slight shift towards higher nystatin concentrations was detected in the cholesterol-containing membranes. In addition, the nystatin activity was shown to be significantly affected by the ergosterol membrane's molar fraction in a non-proportional manner. The observed tension-pore behavior was interpreted using a theoretical model based on the osmotic phenomena induced by the occurrence of size-selective nystatin pores. The number of nystatin pores for different vesicle behavior was theoretically determined and the role of the different mechanical characteristics of the membrane, i.e., the membrane's expansivity and bending moduli, the line tension and the lysis tension, in the tension-pore formation process was quantified. The sterol-induced changes could not be explained adequately on the basis of the different mechanical characteristics, and were therefore interpreted mainly by the direct influences of the membrane sterols on the membrane binding, the partition and the pore-formation process of nystatin.

1. Introduction

Nystatin is an antimicotic agent that belongs to polyene macrolid antibiotics, a family of compounds characterized by a large lactone ring with three to eight conjugated double bonds. It has a broad spectrum of activity against fungi, but it is mainly used for the topical treatment of mucosal and cutaneous *Candida* infections due to its toxic effects when administered systemically [1,2]. As a pore-forming agent, nystatin could also be capable of translocating different cargo molecules into the cells, which makes it a potential specific drug-delivery agent. The biological activity of the polyenes seems to result from their ability to form barrel-like, membrane-spanning channels in the plasma membrane of antibiotic-sensitive organisms [3–5]. These transmembrane pores have size-selective properties: in the case of nystatin they are only permeable to solutes no larger than glucose [6]. The increase in the plasma-membrane permeability to ions and small molecules causes a disturbance to the cellular electrochemical gradients, which ultimately leads to cell lysis and death [3].

Like many cellular membrane activities [7–9] the polyene mode of action has been shown to be affected by the sterol composition in the membrane [10–12]. These effects are probably associated with the sterol-induced changes in the membrane's structural and dynamic properties, such as the regularity of the membrane's lipid organization, the membrane's free volume and the motility of the membrane's constituents [8–12]. Several studies have reported the tendency of cholesterol to increase the line tension and the membrane-expansivity modulus of lipid membranes [13–19]. The addition of ergosterol into the membranes also results in an increase of the membrane-expansivity modulus and the vesicle lysis tension; however, these membrane-stabilizing effects are smaller than those of cholesterol [20–22]. Additionally, various conceptual models were designed to explain the nature of the sterol-lipid membrane interactions [23–25]. The superlattice models suggest distributions with local maxima at theoretically predictable sterol critical molar fractions [26–28].

A significant impact of sterols on the nystatin mode of action has been shown by studies undertaken, in particular, with cholesterol- and ergosterol-containing membranes [29–31]. Many different molecular mechanisms describing the role of sterols in the pore-formation

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process have been presented, including the formation of specific antibiotic-sterol complexes [32,33] and the influences on the polyene membrane's partition due to the sterol-induced changes in the membrane's lipid organization [34,35]. The studies revealed that the increased regularity of the membrane's lipid organization by the addition of cholesterol inhibits the partition of polyenes into the membrane [11–13]. Ergosterol has an even more pronounced lipid-ordering effect than cholesterol in the bilayers formed with saturated lipids [36–39]; however, these effects are substantially diminished in the case of the membranes consisting of an unsaturated lipid like POPC [39]. This is in accordance with the facilitating influences of ergosterol on the nystatin activity in POPC membranes [40]. Furthermore, it has been shown that nystatin has a tendency to form intra-membrane complexes with ergosterol, which could be a major contributing factor to the increase in the nystatin pore-forming activity [32,33]. The changed fluorescence lifetimes of nystatin indicate the formation of nystatin-nystatin or nystatin-sterol complexes [41]. In addition, it has also been found that polyenes are able to form pores in membranes that do not contain any sterols [29,42,43]. The number of nystatin molecules forming the transmembrane pore is estimated to be between 4 and 12 [5,44], and the measured effective radii are mostly in the range between 0.4 and 0.5 nm [45,46]. The radius of nystatin pores was not found to be influenced by the addition of cholesterol or ergosterol into the membrane [47].

Further theoretical and experimental studies are needed to elucidate the polyene-membrane interaction and to obtain a more precise and comprehensive understanding of the influences of the variations in the membrane's sterol content. Our recent work with sterol-free giant unilamellar vesicles (GUVs) and nystatin has revealed some distinct vesicle behavior patterns [43] that could not be obtained from studies on the bulk properties of the membrane-nystatin interactions for small and large unilamellar vesicles [5,30,31]. The GUVs were chosen because they can be manipulated and observed individually, using a micropipette manipulation technique and a phase-contrast microscopy. Hence, we also apply our approach in the present study, in which we observe the influences of membrane sterols (with a focus on ergosterol and its molar fraction) on the nystatin membrane activity and establish a comparison of the behavior of sterol-free and sterol-containing GUVs. We relate the experimental results to the predictions of the theoretical model based on the osmotic phenomena induced by the occurrence of size-selective nystatin pores. Our primary concern is an understanding of the observed differences in the vesicle behavior in connection with the effects of sterols on the membrane's mechanical properties as well as on the formation of nystatin pores. We discuss the results in the context of the tension-pore formation process and the findings in the literature.

2. Materials and methods

2.1. Preparation of vesicles (GUVs) and nystatin solutions

The GUVs were prepared from POPC and/or POPC-sterol mixtures according to the modified method of Angelova et al. [48]. The ergosterol (Sigma-Aldrich, USA) was added to the POPC (Avanti Polar Lipids, USA) in molar fractions of 15, 30 or 45% and the cholesterol (Avanti Polar Lipids, USA) in a 45% molar fraction. The POPC and/or the POPC-sterol mixtures were dissolved in a 1:1 chloroform-methanol solution, spread over platinum electrodes and vacuum dried. Afterwards, the electrodes were placed in an electroformation chamber filled with 2 ml of 0.2-mol/l sucrose solution. An AC electric field was applied, which was stepwise reduced from a starting value of 1 V/mm and 10 Hz to a final value of 0.1 V/mm and 1 Hz. The formed vesicles containing the sucrose solution were transferred in an isomolar glucose solution and kept there at room temperature. The samples were used within three days of preparation; however, the sterol-containing vesicles were not used earlier than two days after their preparation in order to enable a sufficient level of sterol lateral distribution in the membranes.

A stock solution of 1 mmol/l and a stock suspension of 10 mmol/l nystatin were prepared from the lyophilized nystatin (Fluka, Sigma, USA). Pure methanol was used as a solvent, since nystatin has poor water solubility. They were stored in a dark place at −4 °C. The nystatin solutions with the desired concentrations were prepared immediately prior to the experiment. A stock suspension of the appropriate volume was diluted with a 0.2-mol/l glucose solution and stirred using a vortex. The methanol was maintained at volume fractions under 10% in order to minimize its effects on the phospholipid membrane; the methanol volume fraction only increased by 1% as the nystatin concentration was increased by 100 μmol/l. It should be noted, as an experimental limitation, that the saturation effects of nystatin in the surrounding solution associated with its poor water solubility were detected at nystatin concentrations higher than 600 μmol/l.

2.2. Experimental set-up and procedure

The GUVs were observed with an inverted microscope (IMT-2, Olympus, Japan; objective LWD CDPlan40X, NA = 0.55) using phase-contrast microscopy. The images were continuously acquired with a chilled black-and-white CCD camera (C5985, Hamamatsu, Japan) and stored in a personal computer. The images of the vesicles were kept focused in the equatorial plane during the experimental procedure.

A two-compartment cell was used for the vesicle manipulation [43]. The first compartment was filled with the glucose solution that contained the vesicles. The vesicles with an appropriate size and with no anomalies were selected and transferred into the second, measuring compartment using a micropipette manipulation system. The measuring compartment was filled with nystatin solutions of the desired concentrations. The glucose concentrations were equal to 0.2 mol/l in both the compartments. The nystatin concentration remained practically constant during the transfer due to the much smaller volume of the transferred solution compared to the volume of the measuring compartment. The measurements were performed at a room temperature of 24 ± 1 °C.

Individual unilamellar, nearly spherical vesicles with diameters of 40 ± 20 μm were chosen and transferred in groups of 1–5 vesicles into the measuring compartment with nystatin concentrations of between 10 and 800 μmol/l. The vesicles were observed within the first 60 min after their transfer, depending on the duration of the investigated process. At the beginning of each measuring sequence, the control experiments were performed in an isomolar glucose solution without nystatin in order to test the stability of the prepared GUVs and the influence of the micromanipulation procedure. In addition, the GUVs were exposed to a methanol-glucose solution without nystatin in order to check the influence of methanol. The methanol volume fraction was 6% that is our maximal methanol concentration used in experiments with nystatin.

2.3. Image analysis

The images were evaluated qualitatively and quantitatively [43]. Qualitatively, the characteristic vesicle behavior patterns were determined at different nystatin concentrations based on a continuous observation of the phase-contrast images. Quantitatively, the images were assessed in terms of the brightness profile along the line across the vesicle membrane in a radial direction, which was fitted in the halo region by a Gaussian curve [49]. The halo intensity was determined at discrete time intervals and used as a measure of the changing sucrose-glucose ratio inside the vesicle. All the images were analyzed using in-house-produced software, while the quantitative results were evaluated using standard statistical procedures.

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