Filipin-Induced Lesions in Planar Phospholipid Bilayers Imaged by Atomic Force Microscopy

Nuno C. Santos,*# Evgeny Ter-Ovanesyan,§ Joseph A. Zasadzinski,§ Manuel Prieto,* and Miguel A. R. B. Castanho**

*Centro de Química-Física Molecular, Complexo I, Instituto Superior Técnico, 1096 Lisboa Codex, Portugal; #Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1700 Lisboa, Portugal; and §Department of Chemical and Nuclear Engineering, University of California, Santa Barbara, California 93106 USA

ABSTRACT Filipin is a macrolide polyene with antifungal activity belonging to the same family of antibiotics as amphotericin B and nystatin. Despite the spectroscopy and electron microscopy studies of its interaction with natural membranes and membrane model systems, several aspects of its biochemical action, such as the role of membrane sterols, remain to be completely understood. We have used atomic force microscopy (AFM) to study the effect of filipin on dipalmitoylphosphatidylethanolamine bilayers in the presence and absence of cholesterol. The bilayers were prepared by Langmuir-Blodgett deposition over mica and imaged under water. It was shown that filipin-induced lesions could only be found in membranes with cholesterol. In close agreement with electron microscopy results, we have reported the presence of densely packed circular protrusions in the membrane with a mean diameter of 19 nm (corrected for convolution with AFM tip) and 0.4 nm height. Larger circular protrusions (90 nm diameter and 2.5 nm height) and doughnut-shaped lesions were also detected. These results demonstrate that filipin-induced lesions in membranes previously observed by electron microscopy are not biased by artifacts resulting from sample preparation. Filipin aggregates in aqueous solution could also be imaged for the first time. These polydisperse spherical structures were observed in samples with and without cholesterol.

INTRODUCTION Filipin, a polyene antibiotic extracted from Streptomyces filipinensis, belongs to the same family of antifungal agents with low antibacterial activity, such as amphotericin B and nystatin (e.g., Bolard, 1986). They are characterized by a macrolide structure with an amphipathic nature (Fig. 1). The biochemical mechanism of the cellular leakage promoted by filipin is not completely understood. Since the work of Kinsky et al. (1966) the presence of sterols in the membrane is considered essential for the filipin interaction with membranes. However, more recently several works have ruled out this idea (Castanho et al., 1992; Castanho and Prieto, 1992; Milhaud, 1992). The study of the lesions due to the presence of filipin in natural membranes and membrane model systems by electron microscopy began with the work of Kinsky et al. (1966). Soon it became well established that these lesions could only be found in sterol-containing membranes (Tillack and Kinsky, 1973). These works lead to a large use of filipin as a probe for the presence and heterogeneity of the distribution of sterols in membranes (Elias et al., 1979). A tentative systematization of the biochemical action of filipin in the presence and absence of sterol has recently been presented (Castanho and Prieto, 1995).

The use of atomic force microscopy (AFM) (Binnig et al., 1986) in the study of samples of biological interest has become more and more important over the last few years (for reviews see, e.g., Shao et al., 1996; Hansma and Hoh, 1994). Several AFM studies have dealt with planar phospholipid membrane model systems, obtained by Langmuir-Blodgett deposition or vesicle fusion on solid substrates. However, they have had diverse purposes, namely as templates for the imaging of membrane proteins (Singh et al., 1996; Yang et al., 1993), clustering of channel-forming peptides (Mou et al., 1996), alcohol-induced interdigitation of phospholipids (Mou et al., 1994), structure and stability of the membrane (Hui et al., 1995), and phospholipid ordering itself (Zasadzinski et al., 1991; Schwartz et al., 1992). In the present work, AFM was used to study the lesions induced by filipin in planar phospholipid membrane model systems, prepared on fresh cleaved mica in a Langmuir-Blodgett trough, in the presence and absence of cholesterol.

MATERIALS AND METHODS Filipin complex (mostly filipin III; Fig. 1) was purchased from Sigma (St. Louis, MO); cholesterol and dipalmitoylphosphatidylethanolamine (DPPE) were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Langmuir-Blodgett films were prepared on a NIMA trough (Coventry, England), using a subphase of water from a Milli-Q system (Bedford, MA). Mica substrates were rinsed with ethanol and cleaved immediately before immersion in the subphase. Each DPPE/cholesterol mixture, dissolved in chloroform, was gently spread at the air-water interface, and the solvent was allowed to evaporate. Surface pressures from 25 to 40 mN/m were used for the transfer of the two layers, with a waiting time between layers of 3 min to drain away excess water. The layers were kept under water during transfer to petri dishes, where they were incubated overnight in the dark with filipin concentrations ranging from 80 to 300 μM. The sample must be kept under water to prevent the disruption of the membrane.
The AFM studies were performed on a MultiMode AFM (Digital Instruments, Santa Barbara, CA). A 16 × 16 μm² scanner was calibrated on a silicon reference sample with a regular series of pits with a period of 10 μm. Both contact and tapping modes were used. The best images were achieved with silicon nitride twin tips (Digital Instruments) with a spring constant of 0.06 N/m and a nominal radius of 5–40 nm. The AFM stage with the sample was allowed to stabilize for 5–10 min to minimize thermal drift. The probe force was kept to a minimal value (1–5 nN) and checked frequently during the study of a sample. Typical scan rates and sizes were 5–10 Hz and 1–5 μm, respectively. All of the studies and sample preparation were carried out at room temperature. Line-by-line square (Figs. 2 and 3) and offset removal (Figs. 5 and 6) fits were subtracted from AFM images to enhance contrast.

RESULTS

The sample preparation method used in this work leads to the formation of uniform bilayers, which are stable for at least 48 h under water. As shown in Fig. 2, the samples (with or without cholesterol) in the absence of filipin present a smooth surface, without any features detectable by the AFM besides the typical pinholes of planar gel-state membranes (Mou et al., 1995). The images of pure DPPE membranes incubated in a 300 μM filipin solution overnight in the dark (to prevent filipin photochemical reactions) were of poor quality, showing large spherical particles with diameters ranging from 300 to 500 nm. These pictures were not stable between scannings. This suggests that there is no linking of these aggregates to the membrane. After the fluid cell was flushed with water these aggregates disappeared, leaving the typical images of membranes without filipin.

Mixed membranes of DPPE/cholesterol 70/30 (w/w) incubated overnight with filipin initially show the same kind of large aggregates imaged in pure DPPE membranes. However, after extensive flushing with water, we obtained stable images of these aggregates in the membrane that were not moved or modified by the AFM tip (large spherical structures in Fig. 3). Fig. 3 shows that smaller circular protrusions are also present in the membrane. Histograms of heights (mean value is 2.5 ± 0.8 nm, n = 140) and diameters (mean value is 94 ± 17 nm, n = 140) for these protrusions are shown in Fig. 4, A and B, respectively. Some areas in the membrane contained even smaller, densely packed protrusions (see Fig. 5). Corresponding height (mean value is 0.4 ± 0.1 nm, n = 100) and diameter (mean value is 23 ± 4 nm, n = 100) histograms are shown in Fig. 4, D and E, respectively. In some rare cases, we observed doughnut-shaped protrusions in tapping mode (see Fig. 6).

FIGURE 1 Molecular structure of the macrolide polyene antibiotic filipin III, the most important component of the mixture extracted from Streptomyces filipinensis.

FIGURE 2 AFM height image of a bilayer of DPPE/cholesterol mixture 70/30 (w/w) before incubation with filipin. The bilayer was deposited on a freshly cleaved mica substrate by two vertical Langmuir-Blodgett dipping steps at a surface pressure of 25 mN/m. The image was taken in contact mode under water. Natural holes in the bilayer are clearly visible.

FIGURE 3 AFM height (A) and force (B) images of a membrane of DPPE/cholesterol mixture 70/30 (w/w) after overnight incubation with filipin. Large spherical structures are aggregates of filipin in aqueous solution. Small bright dots are filipin-induced circular lesions in the membrane. The images were taken in contact mode under water.
The outer diameter of the doughnut-shaped protrusions in Fig. 6 is \( \sim 80 \) nm, and the inner one is \( \sim 25 \) nm.

It is well established that lateral dimensions in the AFM images of rough samples are enlarged because of the finite size of the AFM tip (Chicon et al., 1987). One solution to this problem involves coadsorption of gold particles of known size on the sample. Imaging of the gold particles makes it possible to determine the radius of the AFM tip and then to calculate the true lateral dimensions of the features of interest (Vesenka et al., 1993). However, this technique is very difficult to apply to such samples as lipid membranes under water. We tried to estimate lateral distortions occurring during scanning of a spherical protrusion in a bilayer (see Fig. 7). A spherical segment is a good model of the protrusion, as the protrusions have very small height/diameter ratio. However, it should be stressed that this spherical segment is only considered for the sake of simplicity. The overall geometry of the filipin-induced lesions cannot be spherical because of the small thickness of the bilayer. In this model \( R_{tip} \) is the radius of curvature of the AFM tip, \( h \) is the height of the protrusion, \( s \) is the curvature radius of the putative sphere, and \( r \) and \( r_{AFM} \) are the real and apparent radii of the protrusion (segment on the surface), respectively. Using the Pythagorean Theorem for triangles ABC and DEC, it is easy to express \( r \) as

\[
r = \sqrt{r_{AFM}^2 - 2R_{tip}h}
\]

(1)
In our case, $h \ll r_{\text{AFM}}$. Therefore, distortions due to the finite tip shape are smaller than in a much more common case of spherical particles on the surface. However, unlike the case of spherical particles (Markiewicz et al., 1994), Eq. 1 does not allow us to estimate $R_{\text{tip}}$ from $r_{\text{AFM}}$ and $h$. According the specifications of the manufacturer, $R_{\text{tip}}$ can vary from 5 to 40 nm for oxide-sharpened twin tips. We calculated $r$ with Eq. 1 with $R_{\text{tip}} = 40$ nm to get the most conservative estimate. Fig. 4, C and F, shows corrected histograms of diameters for large and small protrusions, respectively. The mean values decrease from 94 ± 17 nm to 90 ± 17 nm for the large protrusions and from 23 ± 4 nm to 19 ± 5 nm for the small ones.

Membranes of DPPE/cholesterol mixture with a smaller percentage of cholesterol were also studied (80/20, 90/10, and 95/5, w/w). With decreasing cholesterol concentration, the complexes still appear in the membrane, but the density of their distribution decreases. There is no apparent change in the results caused by decreasing the filipin concentration used in the incubation of the sample from 300 to 80 μM.

DISCUSSION

Despite the fact that DPPE/cholesterol planar membranes incubated with filipin have not been studied so far, our results are consistent with those obtained by negative stain, thin section, and freeze-fracture electron microscopy for several other kinds of membranes (for a review see, e.g., Miller, 1984). These authors have also reported nearly circular protrusions in the membrane (most of the time incorrectly named “spherical”), with mean diameters from 15 to 25 nm. These electron microscopy results correspond exactly to our data on small, densely packed lesions with a mean diameter of 19 ± 5 nm. Depending on the nature of the membrane and experimental procedure, larger protrusions measuring as much as 150 nm across were also reported (Tillack and Kinsky, 1973). Information on the height of these complexes is very scarce, but a tentative value of 5 nm was estimated by electron microscopy (Elias et al., 1979). However, the lower mean value obtained in this work can be considered as the first reliable information on the height of protrusions. Doughnut-shaped protrusions similar to the ones detected in some of our studies have also been reported by some authors using electron microscopy. The dimensions reported are 7–17 nm for the inner diameter and 25–40 nm for the outer diameter, depending on the nature of the membrane (Tillack and Kinsky, 1973; Kitajima et al., 1976).

In agreement with the electron microscopy results, we could also see that these structures cannot be found in bilayers without sterol, assuring us that these are the same filipin-cholesterol complexes that were studied by electron microscopy and spectroscopic techniques (e.g., Bolard, 1986). However, this does not mean that filipin has no biological action in membranes without sterol, but only that there are no lesions or complexes in the membrane that are detectable by microscopy.

The variance of the dimensions of some of the filipin-induced lesions found in our study when compared with some previous works by others might be explained by the different composition of membrane used. As can easily be seen when examining the values found in the literature for several membrane compositions, large differences in size are usually obtained (e.g., Tillack and Kinsky, 1973; Kitajima et al., 1976).

The poorly defined, large spherical particles (Fig. 3) that appear over the membrane in samples of pure DPPE and DPPE/cholesterol mixtures before flushing with water are probably aggregates of filipin in aqueous solution loosely associated with the membrane. Studies of these aggregates are scarce, and they are imaged for the first time in the present work. In agreement with our observation, static and dynamic light scattering spectroscopy studies (Castanho et al., 1994) also indicate a similar polydisperse distribution of sizes and suggest a globular percolation cluster structure.

In a previous work (Castanho and Prieto, 1995) we proposed that the biochemical action of filipin was ruled by two main aspects: filipin concentration and the lateral organization of the sterols in the membranes. Filipin concentration is a very relevant issue because it determines whether filipin is self-aggregated or not (an apparent critical micelle concentration was detected; Castanho et al., 1992; Castanho and Prieto, 1992). A putative interaction of the aggregates themselves with the lipid matrix was considered. In the limit, this interaction could result in lipid “solubilization,” i.e., filipin would act as a detergent. The detection of filipin aggregates loosely adsorbed at the membrane surface reinforces our previous model of filipin biochemical action (Castanho and Prieto, 1995).

The present work shows for the first time that the filipin-induced lesions in sterol-containing membranes previously observed by electron microscopy are not due to any artifact resulting from sample preparation. It should be stressed that AFM images the sample directly in an aqueous environment, with no need for any treatment of the planar lipid membrane. Nevertheless, this conclusion does not overcome the drawbacks of using filipin as a probe for the location of sterols in membranes (Miller, 1984). The use of filipin as a probe is confined to the detection of the presence or absence of the sterol in a certain membrane. Moreover, the usefulness of filipin is limited to techniques that are able to detect the presence of the lesions instead of filipin itself. Even so, the threshold detection limit is unknown, which may be misleading. Most fluorescence techniques, including microscopy, detect the intrinsic presence of filipin and are not adequate for this purpose, because filipin is incorporated into sterol-free membranes (Castanho and Prieto, 1992). As demonstrated in this work, AFM can be used for such a purpose, combining economy of time and simplicity of operation, and elimination of sample preparation artifacts.

Filipin has a peculiar biochemical action at the membrane level, if compared with other polyene antibiotics such as nystatin and amphotericin B. Whereas these last two antibiotics form pores across the phospholipid bilayer, there is
no formation of pores in membranes with filipin, as was shown by permeability studies (for a review see, e.g., Bolard, 1986). Filipin forms large, ordered, supramolecular structures. However, the bilayers remain intact at the filipin concentrations used in this work. Leakage of cellular components during lysis probably occurs by microscopic defects in the highly perturbed lipid matrix.

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