Real-Time Analysis of the Effects of Cholesterol on Lipid Raft Behavior Using Atomic Force Microscopy

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ABSTRACT Cholesterol plays a crucial role in cell membranes, and has been implicated in the assembly and maintenance of sphingolipid-rich rafts. We have examined the cholesterol-dependence of model rafts (sphingomyelin-rich domains) in supported lipid monolayers and bilayers using atomic force microscopy. Sphingomyelin-rich domains were observed in lipid monolayers in the absence and presence of cholesterol, except at high cholesterol concentrations, when separate domains were suppressed. The effect of manipulating cholesterol levels on the behavior of these sphingomyelin-rich domains in bilayers was observed in real time. Depletion of cholesterol resulted in dissolution of the model lipid rafts, whereas cholesterol addition resulted in an increased size of the sphingomyelin-rich domains and eventually the formation of a single raftlike lipid phase. Cholesterol colocalization with sphingomyelin-rich domains was confirmed using the sterol binding agent filipin.

INTRODUCTION

Cholesterol is an essential component of eukaryotic cell membranes, and plays numerous roles in membrane function (Simons and Ikonen, 2000). Recently, it has been suggested that cholesterol is involved in the assembly and maintenance of sphingolipid-rich microdomains or "rafts," which are proposed to act as platforms for the preferential sorting of proteins (Simons and Ikonen, 1997). The raft hypothesis is based on the observation that detergent-resistant membranes, which are enriched in sphingolipids and cholesterol, can be isolated using cold non-ionic detergents (Brown and London, 1998, 2000; Brown and Rose, 1992).

Model membrane studies have shown that lipid-lipid interactions are sufficient to induce the formation of raftlike domains (Dietrich et al., 2001a; Saslowsky et al., 2002). It is well established that phase separation can occur in binary lipid mixtures consisting of lipids that have different phase transition temperatures. Typically, a gel phase, which is characterized by tightly-packed lipids that have limited lateral mobility, co-exists with a fluid or liquid-disordered phase in which the lipids are loosely packed and have a high degree of lateral mobility. Addition of cholesterol has been reported to modify the gel phase component of such systems resulting in the so-called liquid-ordered phase in which the lipids are still tightly packed but acquire a relatively high degree of lateral movement (Sankaram and Thompson, 1990). Lipid rafts are proposed to exist in a state similar to the liquid-ordered phase surrounded by a fluid lipid matrix.

To investigate lipid raft characteristics in model membranes, sphingomyelin (SM) is commonly combined with a fluid-phase lipid, such as dioleoylphosphatidylcholine (DOPC), and cholesterol. SM lipids typically have long, saturated acyl chains that facilitate close packing, an important feature of lipid raft organization (Ahmed et al., 1997; Brown and London, 2000). For this reason, SM-enriched domains in a bilayer are thicker than areas enriched in more fluid, unsaturated lipids, which have kinked chains that effectively shorten the molecules. SM lipids also have significantly higher phase transition temperatures than phosphocholine lipids (e.g., the transition temperature of brain SM is 37–41°C). In addition, there is a favorable interaction between SM and cholesterol, and there is strong evidence that they are colocalized in cell membranes, where cholesterol is thought to promote the formation and stability of lipid rafts (Simons and Ikonen, 2000; Slotte, 1999). The SM/cholesterol interaction is most likely strengthened by hydrogen bonding between the 3′-OH group of cholesterol and the amide of the SM head group (Bittman et al., 1994). Currently, the requirement for cholesterol in raft formation is unclear. For instance, it has been reported that raft domains disappear after cholesterol depletion from the plasma membrane in experiments using cultured cells (Cerneus et al., 1993; Ilangumaran and Hoessli, 1998). However, cholesterol-independent raft domains have also been reported in both model membranes (Milhiet et al., 2002; Saslowsky et al., 2002), and in the brush border membrane of enterocytes (Hansen et al., 2001).

In vitro studies of lipid raft behavior have mainly used fluorescence microscopy to monitor the distribution of fluorescent raft markers (Dietrich et al., 2001a,b; Samsonov et al., 2001; Wang et al., 2000). However, recently, the direct visualization of raftlike domains in model membranes has been achieved using atomic force microscopy (AFM; Milhiet et al., 2001, 2002; Rinia et al., 2001; Saslowsky et al., 2002). AFM is a particularly suitable technique for studying supported lipid layers because of its ability to discriminate Ångstrom-scale height differences between lipid domains, and also to visualize surfaces under aqueous conditions (Dufrêne et al., 1997). Previously, AFM has been used to investigate model raft domains at a number of fixed

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cholesterol concentrations. These studies have provided useful information about the effect of cholesterol on raft thickness and area. In the present study, we have used AFM to analyze in real time the effects of manipulating cholesterol levels in supported model membranes containing DOPC and SM. In addition, we have used the cholesterol binding agent filipin, to reveal the lateral distribution of cholesterol in supported lipid bilayers containing model rafts.

MATERIALS AND METHODS

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), brain sphingomyelin (SM), and cholesterol (Avanti Polar Lipids, Birmingham, AL, USA) were used as received. Methyl-β-cyclodextrin (MβCD), water-soluble cholesterol (MβCD loaded with cholesterol) and filipin complex (minimum 75% filipin III) were purchased from Sigma (UK). Water was obtained from a Millipore water purification system.

Formation of supported lipid monolayers

Lipid monolayers were formed by the Langmuir-Blodgett method using a Nima Series 2011 trough (Coventry, UK). Lipid solutions consisting of SM/DOPC (1:1 mol/mol) with varying amounts of cholesterol were prepared in chloroform at a total lipid concentration, including cholesterol, of 1 mg/ml. To prepare lipid monolayers for transfer onto a freshly cleaved mica support (Goodfellow, Huntingdon, UK), the lipid solution was deposited onto the air-water interface of the Langmuir-Blodgett trough using water as the subphase. Monolayers were compressed to a surface pressure of 30 mN/m, released, and then recompressed three times, and allowed to rest for 10 min on the third compression before being transferred to mica at 10 mm/min. Cholesterol extraction experiments were performed using a subphase containing 10 mM MβCD dissolved in water.

Formation of supported lipid bilayers

Vesicles were prepared by combining DOPC, SM, and cholesterol from chloroform stocks. The chloroform was evaporated under a stream of nitrogen gas and the lipids were rehydrated overnight in water to give a total concentration of 2 mg/ml. The lipid mixture was then vortexed to produce large multilamellar vesicles from which small unilamellar vesicles were prepared by sonicating in a heated (50°C) bath sonicator (Decon Laboratories, Hove, UK) for 30 min. Supported lipid bilayers were formed by depositing 10 μl of vesicle solution followed by 50 μl of buffer (100 mM NaCl, 50 mM Hepes, 2 mM $CaCl_2$, pH 7.6; HBS) onto mica. After 3–5 min incubation at room temperature (22°C), the sample was gently rinsed with the same buffer and transferred to the AFM.

Filipin treatment of supported lipid bilayers

Supported lipid bilayers were preformed on a mica substrate and treated with filipin complex (100 μM) diluted from a dimethyl sulfoxide stock (final dimethyl sulfoxide concentration 1.3% v/v). Incubation of the bilayer with filipin was conducted at room temperature in the dark for 30 min. The samples were gently rinsed with buffer before imaging.

Atomic force microscopy

A Nanoscope III Multimode AFM (Digital Instruments, Santa Barbara, CA, USA) equipped with a J-scanner was used for all imaging. Monolayers were imaged in air in either contact mode using oxide-sharpened DNP-S cantilevers with a spring constant of 0.06 N/m (Digital Instruments) or tapping mode using silicon cantilevers (NCH Pointprobes, Nanosensors, Wetzlar-Blankenfeld, Germany). Supported lipid bilayers were imaged in HBS in fluid tapping mode using oxide-sharpened DNP-S cantilevers with a spring constant of 0.32 N/m (Digital Instruments). For fluid tapping mode imaging the cantilever oscillation was tuned to a frequency between 8–9 kHz and the drive amplitude was adjusted to produce a RMS amplitude of ~0.3–0.5 V. Force was minimized by adjusting the setpoint to just below the jumpoff point of the tip. The scan rate was typically 1–2 Hz. All scanning was carried out at room temperature (22°C). Images were flattened using the Nanoscope III software.

RESULTS

To investigate raft behavior in lipid monolayers, the Langmuir-Blodgett technique was used to transfer monolayers containing SM/DOPC (1:1 mol/mol), with varying amounts of cholesterol, to a mica support for AFM imaging. Separate microdomains were observed in monolayers in the absence of cholesterol (Fig. 1 *A*) and also in monolayers containing 10 and 20 mol% cholesterol (Fig. 1, *B* and *C*), as described previously (Milhiet et al., 2001). The percentage surface area occupied by the thicker SM-rich domains (*lighter gray areas*) increased with increasing cholesterol concentration (24 ± 2%, 33 ± 2%, and 43 ± 6% ($n = 5$) for 0, 10, and 20 mol% cholesterol, respectively). The step height between the DOPC-rich and the SM-rich areas was ~0.5 nm, although this value was strongly dependent on the tip characteristics and whether tapping- or contact-mode AFM was used. At a concentration of 33 mol% cholesterol, the two phases began to coalesce (Fig. 1 *D*), and at 50 mol% cholesterol no distinct domains could be detected (Fig. 1 *E*). To further investigate the relationship between cholesterol concentration and the presence of discrete SM-rich domains, the cholesterol-sequestering agent methyl-β-cyclodextrin (MβCD; 10 mM) was used to extract cholesterol from a monolayer (SM/DOPC 1:1 mol/mol) containing 50 mol%

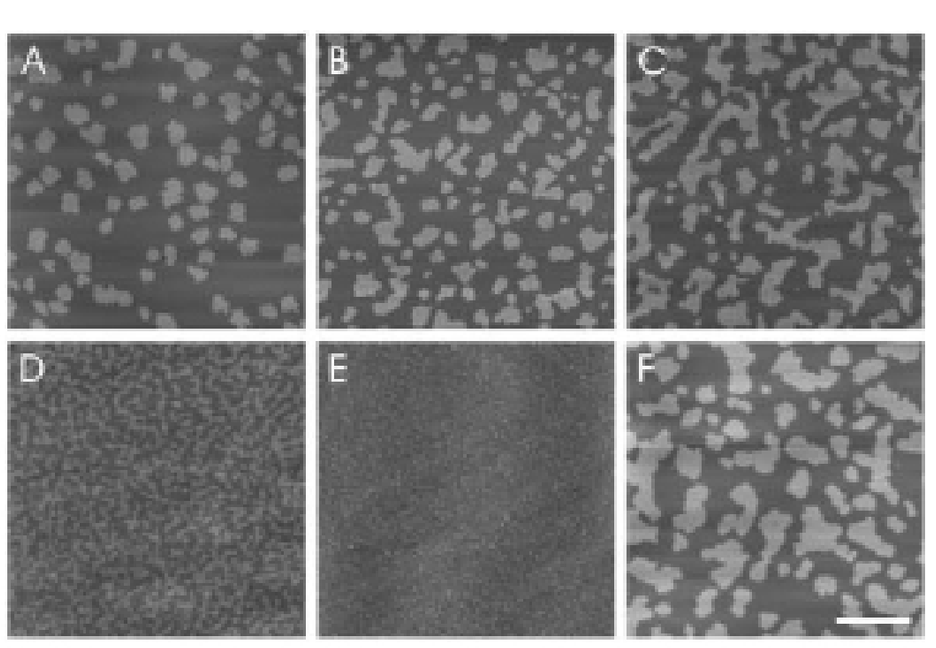

FIGURE 1 Effect of cholesterol on raft behavior in SM/DOPC monolayers. SM/DOPC monolayers (1:1 mol/mol) containing varying amounts of cholesterol were deposited onto mica from the air-water interface using the Langmuir-Blodgett method at a surface pressure of 30 mN/m. (*A*) No cholesterol, (*B*) 10 mol% cholesterol, (*C*) 20 mol% cholesterol, (*D*) 33% mol cholesterol, (*E*) 50 mol% cholesterol, and (*F*) 50 mol% cholesterol monolayer deposited from a subphase containing 10 mM MβCD. Scale bar: 500 nm.

cholesterol. After partial cholesterol depletion from the monolayer (cholesterol was extracted away from the air-water interface into the subphase), SM-rich domains were reformed, and could be visualized after transfer to a mica support (Fig. 1 *F*). In a control experiment, MβCD treatment was found to have no effect on either the lipid domain structure or the surface pressure of a cholesterol-free SM/DOPC monolayer (data not shown). Hence, in our experiments, MβCD was specifically extracting cholesterol.

We next used real-time AFM imaging to investigate the effect of manipulating the cholesterol content of supported lipid bilayers. Initially, bilayers were formed on a mica substrate via vesicle fusion. As with the monolayers, separate domains could be detected in the bilayers (SM/DOPC, 1:1 mol/mol) both in the absence of cholesterol and also at 10, 20, and 33 mol% cholesterol (data not shown). We could not form bilayers successfully from vesicles containing 50 mol% cholesterol. Whereas microdomains in monolayers were of a similar size and occupied a similar area between repeat experiments, microdomains in bilayers exhibited a much greater variation in both these attributes. Typically, the SM-rich domains in bilayers were more irregularly shaped, with a greater variation in size, from the nanometer to micrometer range. It is not known how the microdomains in supported bilayers are related to microdomains in the vesicles before vesicle fusion. For example, a vesicle with a diameter of 50 nm would contribute ~0.008 μm^2 in the supported bilayer. Some domains are much larger than this, indicating that some lipid rearrangement must occur after fusion. However, there was no detectable reorganization or diffusion of the SM-rich microdomains over the time scale investigated, of up to several hours.

When supported bilayers (10 mol% cholesterol/SM/DOPC) were treated with MβCD (10 mM) to deplete them of cholesterol, the SM-rich domains were found to dissolve into the surrounding fluid lipid bilayer (Fig. 2). To confirm that the rearrangement of the bilayer after MβCD treatment was due to cholesterol extraction, a SM/DOPC bilayer containing no cholesterol was treated using MβCD in a similar manner. In this instance, no loss of domains was observed (data not shown). When water-soluble cholesterol (50 μg/ml) was added to preformed bilayers (10 mol% cholesterol/SM/DOPC), the size of SM-rich domains was observed to increase over time, and the height difference between the DOPC-rich and SM-rich regions was reduced from an initial value of 0.7–0.8 nm to a point where separate lipid domains could no longer be detected (Fig. 3). By treating a cholesterol-saturated bilayer (Fig. 4 *A*) with MβCD (20 mM), it was possible to observe the behavior of the bilayer as the cholesterol concentration decreased from high concentrations (no rafts) through to intermediate concentrations, at which separate SM-rich domains reappeared. As the cholesterol concentration decreased further, the domains again dissolved into the surrounding bilayer (Fig. 4 *B*) as in Fig. 2. Unfortunately, it was not possible

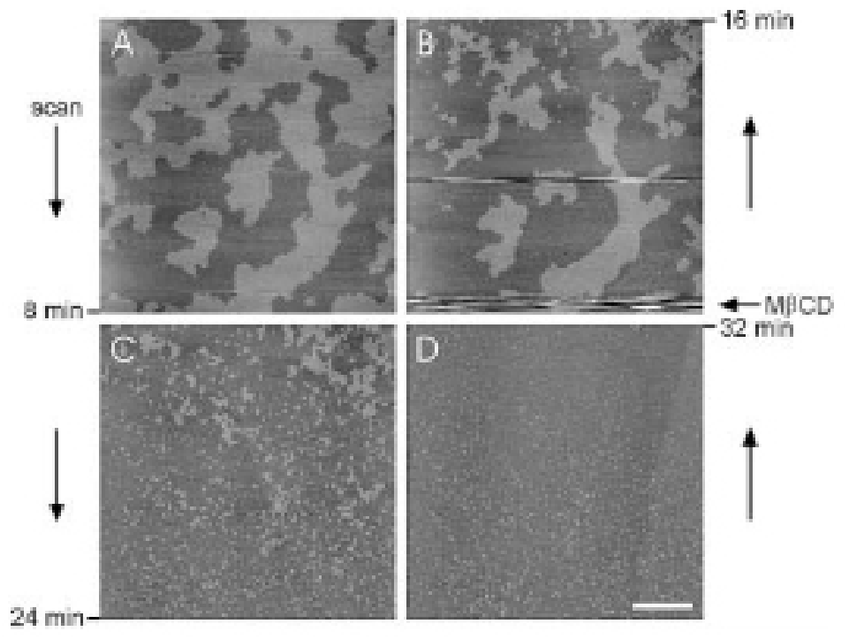

FIGURE 2 Effect of cholesterol depletion on lipid rafts in supported lipid bilayers. (*A*) SM/DOPC (1:1 mol/mol) bilayer containing 10 mol% cholesterol. Lipid rafts can be clearly seen. (*B*) Addition of MβCD (10 mM) at the beginning of the scan resulted in the loss of lipid raft domains in the bilayer. The disturbance at the bottom of the scan shows the point at which the MβCD was injected. Images *B–D* were captured sequentially by scanning over the same area of the sample. The directions of the scans and the times elapsed at the end of each scan are indicated. Scale bar: 1 μm.

to quantitate the cholesterol concentration or the rate of extraction during the AFM imaging.

The cholesterol-binding agent filipin was used to examine the lateral distribution of cholesterol in supported lipid bilayers. Filipin had no effect on supported lipid bilayers that lacked cholesterol (Fig. 5 *A*); however, in bilayers containing 10 mol% cholesterol a highly ordered and striated array could be seen, which was exclusively localized to the SM-rich areas (Fig. 5 *B*). The filipin-induced features protruded

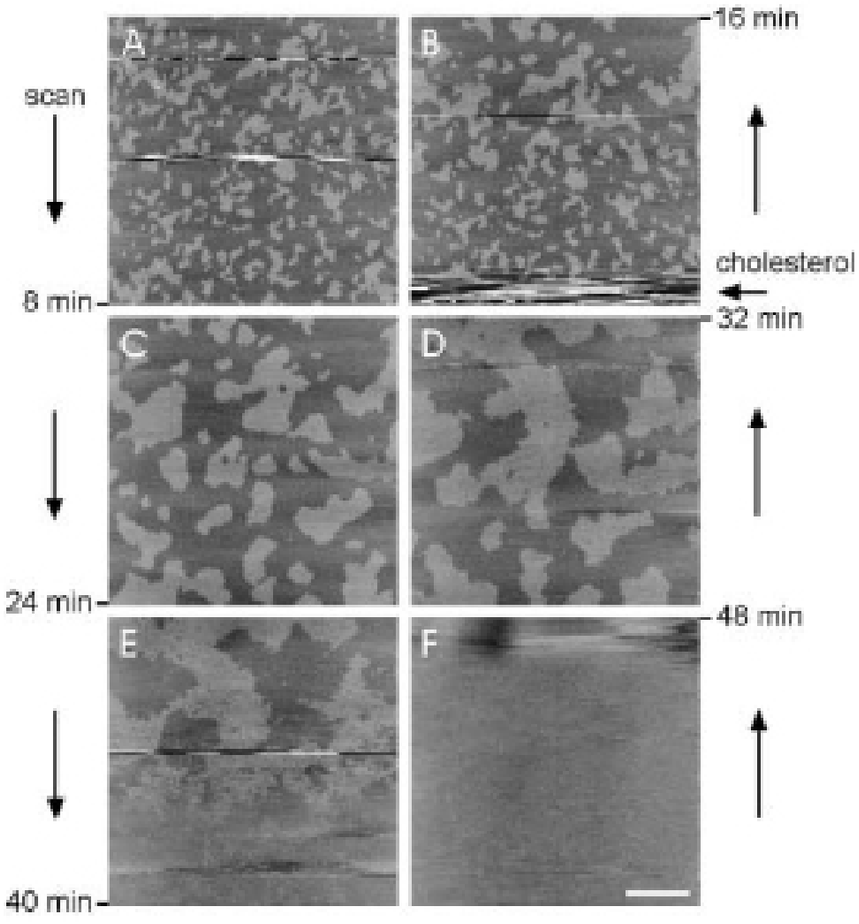

FIGURE 3 Effect of cholesterol addition on lipid rafts in supported lipid bilayers. (*A*) SM/DOPC (1:1 mol/mol) bilayer containing cholesterol (~10 mol%). (*B*) Water-soluble cholesterol (50 μg/ml) was added at the beginning of the scan and the system was allowed to equilibrate briefly before capturing sequential images (*B–F*). The directions of the scans and the times elapsed at the end of each scan are indicated. Scale bar: 1 μm.