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Phase separation in lipid bilayers triggered by low pH

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ABSTRACT

Endocytosis involves the capture of membrane from the cell surface in the form of vesicles, which become rapidly acidified to about pH 5. Here we show using atomic force microscopy (AFM) imaging that this degree of acidification triggers phase separation in lipid bilayers containing mixed acyl chains (e.g. palmitoyl/oleoyl) or complex mixtures (e.g. total brain extract) but not in bilayers containing only lipids with unsaturated chains (e.g. dioleoyl). Since mixed-chain lipids are major constituents of the outer leaflet of the plasma membrane, the type of phase separation reported here might support protein clustering and signaling during endocytosis.

1. Introduction

It was proposed some years ago that biological membranes are not uniform, but rather are organized laterally into various domains [1,2]. Sphingomyelin (SM)- and cholesterol-enriched liquid-ordered domains or 'rafts' have attracted particular attention, and it has been suggested that these domains accumulate a subset of mutually interacting proteins [3]. Raft formation has been observed in model membranes using a variety of techniques, including visualization of fluorescently labeled lipids [4] and atomic force microscopy (AFM) imaging [5–7]. Whether similar domains occur in biological membranes has been more controversial (see, for example, Ref. [8]), although novel imaging techniques have provided support for their existence [9,10]. The current view is that liquid-ordered membrane domains are small, transient, and stabilized by membrane proteins [11].

Within the cell, the various membrane compartments are highly dynamic, raising the possibility of variations in lateral organization as membranes flow between compartments. For instance, during the process of endocytosis, membrane is captured from the plasma membrane in the form of vesicles that are rapidly acidified to a pH of about 5, and subsequently delivered to endosomes [12]. In the present study we have used AFM imaging of supported lipid bilayers to address the possibility that phase separation might be pH-dependent. We report that bilayers composed of lipids with mixed acyl chains undergo phase separation in response to acidification to pH 5. We suggest that this behaviour might have functional consequences during endocytosis.

2. Materials and methods

2.1. Formation of liposomes

Dioleoyl-phosphatidylcholine (DOPC), dioleoyl-phosphatidylethanolamine (DOPE), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE), L-α-PC (from chicken eggs) and total brain lipid extract were obtained from Avanti Polar Lipids (Birmingham, AL) as chloroform stocks. They were mixed at appropriate molar ratios and dried under a stream of nitrogen gas. The dried lipids were then rehydrated overnight to a total concentration of 2 mg/ml with BPC water (Sigma, Poole, UK) and sonicated using a probe sonicator at 10 μm until the mixture went clear, indicating the formation of unilamellar vesicles.

2.2. Sample preparation and AFM imaging of supported lipid bilayers

Liposomes (50 μl of a 2 mg/ml suspension) were allowed to adsorb to freshly-cleaved mica discs (Agar Scientific, Stansted, UK) for 10 min, with addition of 50 μl of buffer solution at either pH 7 (Hepes-buffered saline (HBS) containing 1 mM Ca^{2+}), or pH 5 (0.1 M sodium acetate without Ca^{2+}). Unadsorbed liposomes were washed away, and the supported lipid bilayer was imaged under 150 μl of the same incubation buffer. AFM imaging was carried out using a Veeco MultiMode instrument equipped with a NanoScope IIIa controller. NSC-18 cantilevers (Mikromasch) were used, which had a 30–35 kHz resonant frequency in water, a 3.5 N/m spring constant and a Cr–Au coating. Where appropriate, buffer

Abbreviations: AFM, atomic force microscopy; DOPC, dioleoyl-phosphatidylcholine; DOPE, dioleoyl-phosphatidylethanolamine; HBS, Hepes-buffered saline; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine; SM, sphingomyelin.

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changes were performed in the imaging chamber by exchanging the imaging buffer with four times the volume of the new buffer.

3. Results

The major lipids of the extracellular leaflet of the plasma membrane are the phospholipids PC and PE, along with SM and cholesterol [13]. Since the behaviour of SM- and cholesterol-enriched domains has already been extensively studied [5–7], we began our study by focusing on the behaviour of PC and PE. We attempted to mimic the buffer conditions existing at the cell surface and in endosomes. Specifically, for our 'extracellular' buffer, we used HBS, pH 7, containing 1 mM Ca^{2+}, and for our 'endosomal' buffer we used 0.1 M sodium acetate, pH 5 without Ca^{2+}. Ca^{2+} was omitted since there is evidence that Ca^{2+} is rapidly transported out of the lumen of endocytotic vesicles [14].

A mixture of POPC/POPE (3:1) at pH 7 showed a smooth featureless bilayer with gaps, revealing the mica support (Fig. 1A, top left-hand panel). When the pH was lowered to 5, raised domains in the bilayer (seen as lighter-shaded areas) formed progressively. The first image (Fig. 1A, top-center panel) was taken at time zero, and the same area was subsequently scanned repetitively over 240 min. New domains formed, and existing domains grew over time. The size of the domains varied, but at later times the larger ones were about 500 nm in diameter. After 240 min, the raised domains accounted for about 50% of the total bilayer area. A section through the bilayer (Fig. 1B) showed that the domains were about 0.7–0.9 nm higher than the background bilayer.

To determine if the phase separation observed was reversible, we made POPC/POPE bilayers at pH 5, changed the pH to 7 and imaged the same area over 100 min. We found that the raised lipid domains generated at pH 5 did not disappear over time (Supplementary Fig. S1). In fact, they continued to form, and occupied over 50% of the total bilayer area after 100 min. This result suggests that the initial reduction in pH triggered a process that was irreversible, even when the pH was returned to 7.

Given the well-known effects of Ca^{2+} on the properties of lipid bilayers (e.g. Refs. [15–17]), it was important to exclude the possibility that it was the removal of Ca^{2+} from the buffer, rather than the reduction in pH, that was causing the domain formation. We therefore repeated the experiment illustrated in Fig. 1 except using phosphate–citrate buffers at pH 7 and pH 5 without Ca^{2+}. We observed that domains were absent at pH 7 (Supplementary Fig. S2A) but appeared when the pH was lowered to 5 (Supplementary Fig. S2B). Interestingly, the domains tended to be fewer and larger than those seen on switching from HBS, pH 7, plus Ca^{2+} to sodium acetate, pH 5, minus Ca^{2+}, suggesting that Ca^{2+} was having an effect on the behaviour of the lipid domains.

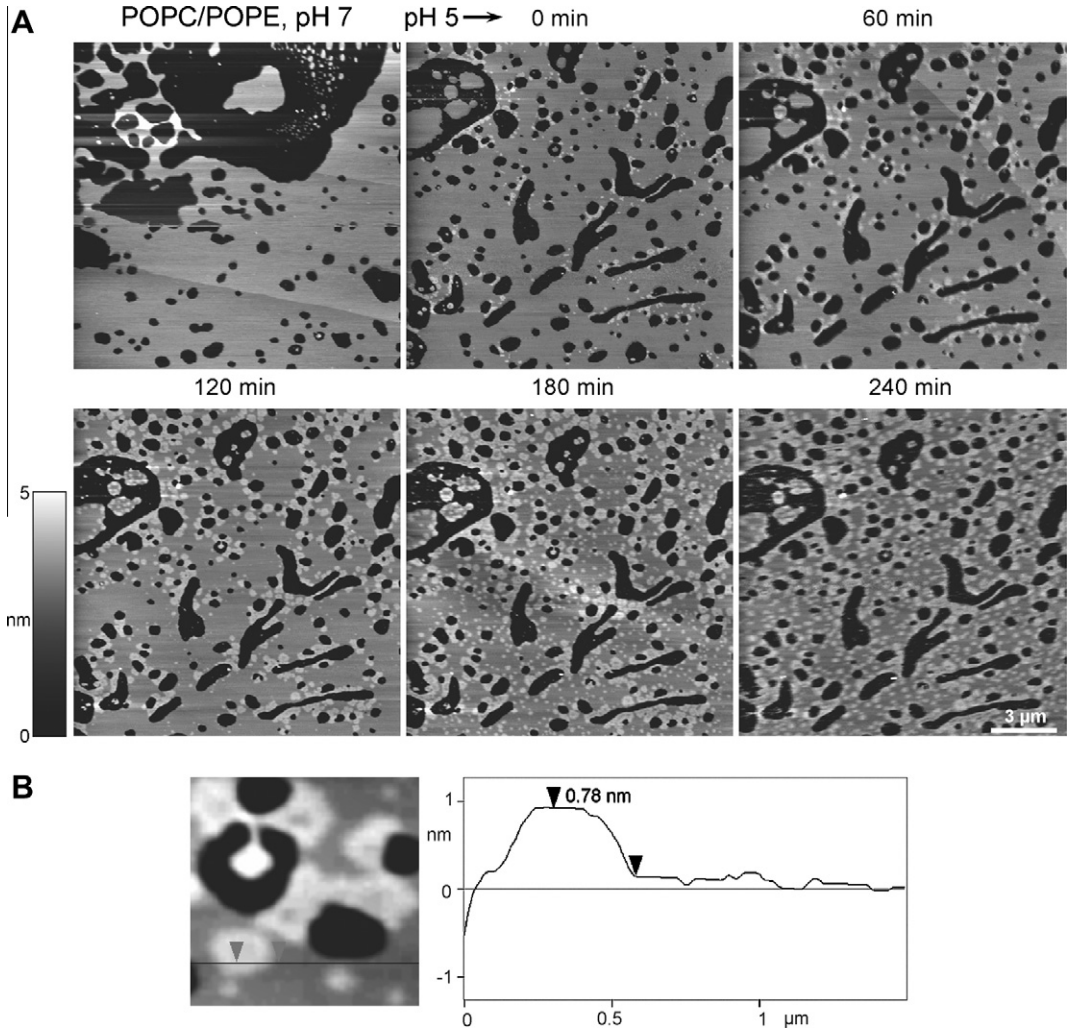

Fig. 1. Induction of lipid domains in POPC/POPE bilayers in response to lowering of pH. (A) The top-left panel shows an AFM image of a supported bilayer composed of POPC/POPE (3:1) at pH 7. The other panels show the same sample at various times after lowering the pH to 5. Note the increase in size and number of lipid domains over time. A shade-height scale is shown at the left. (B) Profile of a lipid domain.