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Cholesterol stabilizes fluid phosphoinositide domains

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ABSTRACT

Local accumulation of phosphoinositides (PIPs) is an important factor for a broad range of cellular events including membrane trafficking and cell signaling. The negatively charged phosphoinositide headgroups can interact with cations or cationic proteins and this electrostatic interaction has been identified as the main phosphoinositide clustering mechanism. However, an increasing number of reports show that phosphoinositide-mediated signaling events are at least in some cases cholesterol dependent, suggesting other possible contributors to the segregation of phosphoinositides. Using fluorescence microscopy on giant unilamellar vesicles and monolayers at the air/water interface, we present data showing that cholesterol stabilizes fluid phosphoinositide-enriched phases. The interaction with cholesterol is observed for all investigated phosphoinositides (PI(4)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2 and PI(3,4,5)P_3) as well as phosphatidylinositol. We find that cholesterol is present in the phosphoinositide-enriched phase and that the resulting phase is fluid. Cholesterol derivatives modified at the hydroxyl group (cholestenone, cholesteryl ethyl ether) do not promote formation of phosphoinositide domains, suggesting an instrumental role of the cholesterol hydroxyl group in the observed cholesterol/phosphoinositide interaction. This leads to the hypothesis that cholesterol participates in an intermolecular hydrogen bond network formed among the phosphoinositide lipids. We had previously reported that the intra- and intermolecular hydrogen bond network between the phosphoinositide lipids leads to a reduction of the charge density at the phosphoinositide phosphomonoester groups (Kooijman et al., 2009). We believe that cholesterol acts as a spacer between the phosphoinositide lipids, thereby reducing the electrostatic repulsion, while participating in the hydrogen bond network, leading to its further stabilization. To illustrate the effect of phosphoinositide segregation on protein binding, we show that binding of the tumor suppressor protein PTEN to PI(5)P and PI(4,5)P_2 is enhanced in the presence of cholesterol. These results provide new insights into how phosphoinositides mediate important cellular events.

1. Introduction

Phosphoinositides (diacylglycerophosphatidylinositolphosphates) have been shown to mediate a large variety of important physiological functions by influencing the activity and/or localization of membrane associated proteins (Shewan et al., 2011). Several of these phosphoinositide-mediated signaling events are affected by cholesterol levels, and it has been suggested that at least some of these functions are associated with liquid-ordered (l_o) domains that are enriched in sphingolipids and cholesterol (rafts) (Johnson et al., 2008). Studies linking phosphoinositide signaling events to lipid rafts typically fall into one of these categories: cholesterol depletion studies that associate decreased cholesterol levels to an activity change of the respective pathway (Das et al., 2010; Fox et al., 2011; Hao and Bogan, 2009; Kwik et al., 2003; Peres et al., 2003; Seveau et al., 2007); studies that extract raft domains and analyze them with respect to protein and/or lipid content (Furt et al., 2010; Gao et al., 2011; Koushik et al., 2013); studies investigating the co-localization of phosphoinositides or phosphoinositide binding proteins with raft markers (Furt et al., 2010; Shen-Tu et al., 2010) or studies that highlight the localization of phosphoinositide modifying enzymes to rafts (Johnson et al., 2008; Varnai and Balla, 2007).

Physiological models suggesting raft residency of phosphoinositides, are faced with the fundamental problem that the stearoyl/arachidonoyl acyl chain composition of naturally

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occurring phosphoinositides is believed to be unfavorable for liquid-ordered environments, such as lipid rafts (Epand et al., 2004a). Using live cell FRET experiments, van Rheenen et al. (2005) strongly questioned the presence of $PI(4,5)P_2$ in lipid rafts and showed that Triton X-100 induces $PI(4,5)P_2$ domain formation. In further support of this argument, *in vitro* studies showed that $PI(4,5)P_2$ does not partition into liquid-ordered domains (Shaw et al., 2006) unless a phosphoinositide-binding protein like GAP-43 is present in the raft (Tong et al., 2008). It is important to stress that these *in vitro* studies utilized lipid compositions characteristic for the outer leaflet of the plasma membrane, which is probably an inadequate model for the characterization of the inner leaflet phosphoinositide partitioning.

Phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) comprises only 1% of the total plasma membrane lipids, and the total cumulative concentration of its protein ligands exceeds its concentration in the cell (Catimel et al., 2008). This suggests that the fine-tuning of phosphoinositide-mediated signaling events requires a tight control of its lateral distribution. Even though raft association of phosphoinositides cannot be unequivocally proven or disproven at this point, lateral accumulation of phosphoinositides in response to specific cellular events has been observed in several instances (Cai and Devreotes, 2011; Calloway et al., 2011; Gao et al., 2011; Golebiewska et al., 2011; Illien et al., 2012; Rogasevskaia et al., 2012). Phosphoinositide accumulation can be triggered through interaction with cationic proteins or peptides (McLaughlin and Murray, 2005) as well as through interaction with cationic calcium (Wang et al., 2012). It has been shown in model systems that cholesterol supports $PI(4,5)P_2$ domain formation (Levental et al., 2009). This paper explores which underlying mechanisms lead to cholesterol-induced $PI(4,5)P_2$ domain formation, and whether the observed cholesterol-induced clustering is also found for the other naturally occurring phosphoinositide derivatives. To extend our previous studies that utilized indirect methods to study phosphoinositide phase behavior (Graber et al., 2012; Kooijman et al., 2009; Redfern and Gericke, 2004, 2005), we use in this study fluorescence microscopy to visualize cholesterol dependent phosphoinositide domain formation in giant unilamellar vesicles as well as monolayers at the air/water interface. We are providing a first glimpse of the phase state of these phosphoinositide domains, and we investigate to what extent the interaction of proteins with model membranes is affected by a heterogeneous phosphoinositide distribution.

2. Experimental

2.1. Materials

L-α-Phosphatidylinositol (PI) (liver, bovine) (sodium salt), L-α-phosphatidylinositol-4-phosphate (PI(4)P) (brain, porcine) (ammonium salt), L-α-phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) (brain, porcine) (ammonium salt), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-myo-inositol-3′,4′-bisphosphate) ($PI(3,4)P_2$) (ammonium salt), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-(1′-myo-inositol-3′,5′-bisphosphate) ($PI(3,5)P_2$) (ammonium salt), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-(1′-myo-inositol-3′,4′,5′-trisphosphate) ($PI(3,4,5)P_3$) (ammonium salt), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (SAPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (RhBDOPE), 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC), 1-oleoyl-2-{6-[4-(dipyrromethene boron difluoride)butanoyl] amin}hexanoyl-sn-glycero-3-phosphoinositol-4,5-bisphosphate (ammonium salt) (Topfluor $PI(4,5)P_2$) were obtained as powders from Avanti Polar Lipids (Alabaster, AL) and were used as received. Stock solutions of the lipids were obtained by dissolving the respective phosphoinositide in a 20:9:1 mixture of chloroform, methanol and water, while the less polar lipids like POPC were dissolved in a 2:1 mixture of chloroform and methanol. The concentrations of the respective stock solutions were determined using a phosphate assay (Rouser et al., 1970). All C6-BODIPY TMR labeled phosphoinositides (excitation: 542 nm, emission: 574 nm) were purchased from Echelon Biosciences Inc. (Salt Lake City, UT). 1-[2-Hydroxy-3-(N,N-di-methyl-N-hydroxyethyl) ammoniopropyl]-4-[β-[2-(di-n-butylamino)-6-napthyl] vinyl] pyridinium dibromide (Di-4-ANEPPDHQ) was kindly provided by Dr. Robert Twieg (Kent State University, Kent, OH). Boron dipyrromethene difluoride (BODIPY) cholesterol was provided by Dr. Robert Bittman (City University of New York, New York, NY). All salts and buffers used for this study were of enzyme grade and were obtained from Fisher Scientific (Fairlawn, NJ). Chloroform (99.9%), water (HPLC grade) and methanol (99.9%) were also obtained from Fisher Scientific (Fairlawn, NJ).

2.2. Preparation of recombinant PTEN

The cDNA encoding full-length (1–403) human PTEN was cloned into the *NdeI* and *XhoI* sites of the pET30b vector (Novagen), thereby, introducing a 6-histidine tag at the C-terminus. PTEN proteins were expressed in *Eschericia coli* BL21(DE3) cells. To increase protein solubility, cells were grown at 37 °C until the OD at 560 nm reached 0.5–0.6. The culture was shifted to 21 °C and after 30 min, protein expression was induced with 0.05 mM isopropyl-β-D-thiogalactoside. After 20–22 h, the culture was harvested. His_6-proteins were purified with a HisTrap HP Kit from GE Healthcare using buffers with 10 mM mercaptoethanol. The PTEN proteins were further purified with a Superdex 200 16/60 column in 100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM dithiothreitol. The final purification was done with a MonoQ 5/5 anion-exchange column in 10 mM Tris, pH 7.4 with a linear gradient of 50–600 mM NaCl. Fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Those fractions containing >95% pure protein were pooled. Dithiothreitol was added to 10 mM, and the proteins were stored on ice because initial experiments revealed that freeze-thawing resulted in protein aggregation. The yield of recombinant protein was 1.5–2.0 mg/l. Phosphatase activity was confirmed as described (Campbell et al., 2003).

2.3. Preparation of giant unilamellar vesicles (GUVs) using the slow rehydration and electroformation methods

With a few changes, we followed the method described in the literature (Akashi et al., 1996; Korlach et al., 1999). Lipid stock solutions were prepared as described above to yield a ~10 mM concentration. 0.2 μmol of stock lipid solution with the desired lipid mixture (see experiments for exact lipid ratios) was mixed with 0.1 mol% of the respective fluorescently labeled lipid and diluted with 300 μl organic solvent (chloroform/methanol, 2:1, v/v). The sample was placed in a home-made test tube (internal diameter 1.5 cm) and the solvent was evaporated at 50 °C with a rotary evaporator (Buchi, Rotavapor R-205) to form a thin lipid film on the surface of the lower portion of the tube (~2–3 cm height). The tube was subsequently placed in a vacuum oven for >4 h to remove remaining traces of organic solvent. The completely dried lipid film was then prehydrated at 50 °C with water-saturated nitrogen gas for ~30–45 min. Subsequently, 2 ml N_2 purged aqueous solution (pH 7 buffer made from 100 mM NaCl, 5 mM PIPES, and 0.1 mM EDTA, NaOH and HCl were used to adjust the pH) were added gently to the test tube. The tube was sealed under nitrogen and wrapped