pH-dependent formation of membranous cytoplasmic body-like structure of ganglioside G(M1)/bis(monoacylglycero)phosphate mixed membranes.

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A characteristic feature of endosomes along with the degradative endocytic pathway is the accumulation of vesicles within the organelle (1,2). Recently, it has been shown that the unconventional phospholipid bis(monoacylglycero) phosphate (BMP, also known as lysobisphosphatic acid, LBPA) can induce the formation of multivesicular liposomes that resemble multivesicles endosomes (3). BMP is a structural isomer of phosphatidylglycerol with characteristic sn-1, sn-1’ glycerophosphate stereoreconfiguration (4,5). This lipid is highly enriched in the specific internal membrane domains of multivesicular late endosomes where the lipid comprises >70% of the total phospholipids (6,7). It has been reported that late endosomes/lysosomes change their organization from multivesicular to multilamellar membranes under different pathological conditions and by treatment with certain drugs. These multilamellar vesicles, in which membranes are tightly stacked, are called membranous cytoplasmic bodies (MCB). Although the involvement of BMP domains in late endosomes (8) and lipid-protein interaction (9) have been suggested, the mechanism of the formation of MCB is not well understood. Recently we have shown that a drug that induces multilamellar endosomes alters BMP liposomes from swollen and loosely packed lamellar vesicles to closely stacked multilamellar structures at low pH (10).

Sphingolipidosis is a genetic disease defective in the proteins involved in sphingolipid metabolism (11). Accumulation of MCBs is a characteristic feature of this disease. Different sphingolipids are accumulated depending on the defect. These lipids, such as sphingomyelin and galactosylceramide, themselves form multilamellar structures in aqueous solution. In contrast, in GM1 gangliosidosis, micelle-forming lipid GM1 is extensively accumulated and still MCBs are formed. Therefore, it is of interest to investigate the conditions in which the accumulation of GM1 induces the formation of closely stacked membranes. In our study, we examined the membrane structure of ganglioside/BMP mixture in neutral and acidic pH conditions, the latter of which resembles the lumen of late endosomes/lysosomes.

First, we examined whether the accumulated GM1 colocalize with the BMP-rich membrane domains in intact cells. The addition of exogenous ganglioside to cultured cells mimics the behavior of the cells from gangliosidosis (12). Diffuse fluorescence was observed when cultured human skin fibroblasts were fixed, permeabilized, and labeled with fluorescently labeled cholera toxin, which recognizes GM1 (see Fig. 4 of the Supplementary Material). In contrast, intracellular compartments were brightly labeled with cholera toxin when cells were grown in the presence of 10 μM GM1. The fluorescence was colocalized with that labeled with anti-BMP antibody. The result suggests the presence of BMP and GM1 in the same membrane domains. We next examined the
membrane structure of BMP/GM₁ complex. 2,2′-Dioleoyl-
-sn-1,sn-1′-BMP is a major molecular species of naturally occurring BMP (7,13). We chemically synthesized 2,2′-
dioleoyl-sn-1,sn-1′-BMP (14) and measured the structure of the membranes in the presence of GM₁ by using electron microscopy and small-angle x-ray scattering (SAXS). Fig. 1 shows freeze-fracture electron micrographs of the GM₁/BMP (1:1 mol/mol) mixture at pH 7.4 and 4.6. The particles observed at pH 7.4 were mainly unilamellar vesicles, as demonstrated in cross-fracture images, whereas the results at pH 4.6 indicated structures filled with multiple layers or large multilamellar vesicles. Each layer was closely stacked, and the distance between the adjacent layers was <10 nm. The size of vesicles at pH 7.4 was ~100–300 nm diameter in contrast to ~300 nm–3 μm diameter at pH 4.6. Similar results were observed by negative-staining electron microscopy (data not shown). In Fig. 1, pH dependence of the SAXS patterns of the GM₁/BMP (1:1 mol/mol) mixture are also shown. At pH 8.5–6.5, the SAXS profiles displayed similar curves, exhibiting an evident minimum at \( q = 0.55 \) nm\(^{-1}\) and a broad bell-shaped peak at \( q = 1 \) nm\(^{-1}\). These are characteristics of a scattering curve from an assembly of identical small particles. It is reported that dioleoyl BMP forms a diffuse lamellar structure at a pH range of 3.0–8.5 (10,15), whereas GM₁ forms a stable micellar structure at a pH range of 3.6–8.0 (16). Considering the negatively charged bulky headgroup of GM₁, which gives a high curvature when inserted into the membrane, it is expected that the GM₁/BMP mixture formed such compact aggregates. At pH 5.5, however, the SAXS pattern exhibited two small peaks at \( q = 0.78 \) and 1.56 nm\(^{-1}\) in addition to the broad peak at \( q = 1 \) nm\(^{-1}\). These two peaks correspond to the first- and second-order diffraction peaks from a lamellar structure with an 8.06 nm repeat distance. At pH 4.6, the first- and second-order peaks became much more evident, indicating that the acidic pH condition transformed the GM₁/BMP mixture from small aggregates to a planar lamellar structure. The dose response of GM₁ indicates that the alteration of the membrane structure was inducible by the addition of as low as 10% of GM₁ (see Fig. 5 in the Supplementary Material) at low pH.

One of the consequences of the storage of sphingolipids in MCBs, including GM₁, is the accumulation of cholesterol. It is proposed that the preferential association of sphingolipids and cholesterol causes the accumulation of cholesterol in MCBs (8). We investigated whether the GM₁/BMP membrane traps cholesterol in a pH-dependent manner (Fig. 2). Methyl-β-cyclodextrin (MβCD) extracts cholesterol from the membrane. Extraction of cholesterol from BMP and GM₁/BMP membranes by MβCD was investigated at pH 7.4 and 4.6. Cholesterol was equally extracted from the BMP liposomes irrespective of pH. The presence of GM₁ did not affect the extraction at pH 7.4. In contrast, the extraction of cholesterol was significantly reduced in the presence of GM₁ at pH 4.6. The addition of 10 mol % cholesterol did not alter the gross structure of the GM₁/BMP membranes (data not shown). This result suggests that the formation of the closely packed multilamellar structure of GM₁/BMP in an acidic environment prevents the cholesterol extraction by MβCD.

Fig. 3 shows the examination of the effects of various gangliosides on the membrane structure of BMP at pH 4.6. Similar to GM₁/BMP, lamellar diffraction peaks were observed in GM₂/BMP membrane. However, the GM₃/BMP and GD₃/BMP mixtures did not exhibit clear lamellar peaks, suggesting that a branched carbohydrate chain is required for

![FIGURE 1](image_url)  (Left) Freeze-fracture electron micrographs of GM₁/
BMP (1:1 mol/mol) mixture at different pH. (Right) SAXS patterns of GM₁ at pH 4.6 and GM₁/BMP mixture at different pH.

![FIGURE 2](image_url)  Cholesterol extraction from BMP/cholesterol and GM₁/BMP/cholesterol (10 mol % cholesterol) membranes at different pH.
suggest that BMP and a low pH are additional players in cholesterol accumulation in MCBs.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

REFERENCES and FOOTNOTES


