

Possible roles of sphingolipids in clathrin-mediated endocytosis - Vesicle formation by a glycerophospholipid cycle - a hypothesis

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Abstract

The participation of sphingolipids in non-clathrin-dependent endocytosis has been known for some time. The participation of sphingolipids in clathrin-mediated endocytosis (uptake of transferrin) has only recently been reported. It was shown that not only does the synthesis of sphingolipids play a significant role in clathrin-mediated endocytosis but also the degradation of sphingolipids. Thus, a cycling process seems likely and may suggest a role of lipids in creating vesicles whereas the current discussion gives curvature-inducing and -sensing proteins the leading role in this process.

We present a hypothetical cycle in which serine of phosphatidylserine and 2n-fatty acids of various glycerophospholipids are substrates for sphingolipid synthesis (sphingomyelin). Subsequently, sphingomyelin is broken down via acid sphingomyelinase, ceramidase, sphingosine-1-kinase and sphingosine-1-phosphate lyase. Phosphoethanolamine originating from the last reaction can be used for phosphatidylethanolamine synthesis. Phosphatidylserine synthase changes the head group of phosphatidylethanolamine to yield phosphatidylserine. Thus the original glycerophospholipids are restored.

It is likely that the particular metabolites not only have a physical role but also a role as signal molecules activating step by step the subsequent reaction in the developing vesicle and deactivating the previous one. That means that it is very difficult to measure the only temporarily activated enzymes. In order to prove this hypothesis it is initially important to search for the particular enzyme protein in the coated pit or vesicle independent of its activity. As the clathrin-mediated endocytosis is a fundamental process of all cells and is also involved in various diseases we should understand it not only for theoretical but also for medical reasons

Keywords

Glycerophospholipid cycle, curvature, clathrin-mediated endocytosis, sphingolipids

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Introduction

Until recently sphingolipids have only been thought to play a role in clathrin-independent endocytosis, and not in clathrin-mediated endocytosis (CME) [1]. Transferrin uptake was shown to be independent of sphingolipids. But Meyer et al. [2] provided evidence that sphingolipids are involved in CME (uptake of transferrin) under physiological conditions avoiding

cold preincubations. Myriocin, an inhibitor of the first step of sphingolipid synthesis – serine palmitoyltransferase (SPT) – inhibited CME in L929 and other cells. Not only myriocin but also other inhibitors of sphingolipids inhibited CME. Thus fumonisins B1, an inhibitor of ceramide synthase inhibited CME by about 60 % whereas N-butyldeoxynojirimycin (DB-DNJ), an inhibitor of ceramide-specific glucosyltransferases inhibited CME by about 20 % [2]. Additional information.

Shakor et al. [3] investigated transferrin-mediated cell proliferation in sphingomyelin-deficient lymphoma cells. Both sphingomyelin synthase 1 gene transfection and exogenous short-chain sphingomyelin treatment increased transferrin uptake in these sphingomyelin-deficient cells. Not only enzymes of sphingolipid synthesis influence CME but also a metabolizing enzyme, acid sphingomyelinase does too (A. Wendt, thesis in preparation, our laboratory) implying a cycling process. The participation of sphingolipids in such an important process as the CME deserves to bring forward new ideas as to how these molecules may work.

Sphingolipids play roles in contrary processes in the cell. On the one hand they induce cell cycle arrest, apoptosis, and cell senescence [4,5,6,7,8,9,10,11,12] but on the other hand they support cell growth. According to Hait et al. [9] sphingosine-1-phosphate favours growth whereas ceramide favours cell death (rheostat-theory). The balance between the two possibilities determines the fate of the cell. The participation of sphingolipids in CME opens up an additional possibility to explain the opposite effects of sphingolipids. Signals coming from extracellular space can be either growth promoting or growth inhibiting. Thus the signals from outside may determine – at least partially – which role the participating sphingolipids play. In addition to the question as to whether sphingolipids are the reason for the fate of the cell (apoptosis or growth) or only the transmitter of an outside signal to the signalling into the cell, the possible role, which sphingolipids may directly play in CME needs to be explained. Using our own data and data from literature we present a hypothetical glycerophospholipid cycle, which is able to provide a possible explanation for the interchange of glycerophospholipids into sphingolipids and back to glycerophospholipids. This interchange may help to explain at least to some extent how the needed curvature for vesicle formation from a flat membrane could be induced. In the current discussion curvature-inducing and -sensing proteins account for changes in curvature needed for vesicle formation. Lipids should only play a permissive role [13]. In a review McMahon et al. [14] list all the known proteins and their decisive domains participating in CME and being important for nucleation and vesicle formation. Our hypothesis does not doubt the roles of proteins in CME. For example, endocytosis starts with nucleation [14] induced by N-BAR domain-containing proteins. I propose to consider in the same process the domain-forming properties of

sphingolipids (and cholesterol). Both proteins and lipids play their roles. But contrary to the roles of proteins the roles of lipids in CME are neglected. In the long-term view we have to find out the interplay of lipids and proteins in CME. Here we propose possible roles of lipids especially sphingolipids in CME.

Possible roles of sphingolipids in clathrin-mediated endocytosis

Platform for internalization

Sphingolipids have distinct physical properties compared with other lipids that may be relevant in endocytosis. Van Blitterswijk et al. [15] emphasised that ceramide is not a priori an apoptotic signal molecule but that the conversion of sphingomyelin into ceramide can play a membrane structural (physical) role, with consequences for membrane microdomain function, membrane vesiculation, fusion/fission and vesicular trafficking. Domain formation facilitates budding [16]. Though the sphingolipid ceramide and the glycerolipid diacylglycerol have a similar structure, their behaviour in membranes is quite different. Caused by the additional hydrogen bond and the two long, mostly saturated chains, ceramide and sphingomyelin together with cholesterol increase the order and reduce fluidity of membranes, leading to tighter packing and rigidization. Ceramide tends to self-aggregation in-plane, building up microdomains [15]. This behaviour may explain the disappearance of the dependency of CME on sphingolipids after preincubation at low temperature [2]. For measuring CME it is common to load the transferrin receptors under cold conditions. However, cold incubation may account for missing the participation of sphingolipids in CME [2]. Similar results are reported by Boucrot et al. [17] measuring the dependence of clathrin-dependent receptor-mediated endocytosis of low density lipoprotein (LDL). As long as the cells are maintained under warm conditions (and in complete serum) knockdown of AP-2 strongly inhibits LDL uptake. This dependency disappears after cold preincubation (4 °C). They conclude that under certain stress conditions alternative endocytic structures induce compensatory trafficking pathways. It is a fact that the temperature is a decisive factor changing the physical properties of lipids. At the physiological temperature of 37 °C sphingolipids may be needed for domain formation during endocytosis. At this high temperature

glycerophospholipids are more fluid than sphingolipids whereas at low temperatures glycerophospholipids are also less fluid and may be able to contribute to a platform formation probably needed for a coated pit. Myriocin, the inhibitor of SPT, not only induced changes in sphingolipids but also changes in the glycerophospholipid spectrum in L929 cells [2]: Glycerophospholipids, above all, species of phosphatidylcholine with longer, saturated and alkylated fatty acid species were increased. This may reflect a compensatory lipid spectrum approaching properties of the sphingolipids with their particular physical properties needed in endocytosis.

How curvature may be induced

Apart from building up platforms, sphingolipids could also help to change a flat bilayer into a vesicle. Lipids have very different shapes and structures. They can induce positive and negative curvature in a membrane. Recently this aspect of lipids' properties has been neglected, following the discovery of curvature-inducing and sensing proteins. Kirchhausen [18] summarized the latest experiments showing "that specific assembly of clathrin protein scaffold coupled to the membrane seems to be the most prevalent mechanism for bending a lipid layer in a cell" besides membrane crowding. However, according to our laboratory results, sphingolipid synthesis [2] and breakdown (A. Wendt, thesis in preparation, our laboratory) play a role in CME. Thus a cycling process connected to great changes in the shape and structure of the involved lipids seems likely.

Plasma membrane surface asymmetry can be a driving force for vesicle formation during endocytosis by, for example, the addition of aminophospholipids, which can be flipped to the inner leaflet [19]. Synthesis of lipid molecules at only one leaflet also entails asymmetry, which can cause or facilitate budding. Various lipid-metabolizing enzymes change the shape and structure of a lipid and thus influence curvature of a bilayer:

Phospholipase C: In 1978 Allan et al. [20] already showed that curvature of a membrane can be induced by the action of a phospholipase C: for example, diacylglycerol produced from phosphatidylcholine can easily flip to the other leaflet of the bilayer. Here it can be trapped by diacylglycerol (DAG)-kinase to produce phosphatidate, inducing curvature by the asymmetric distribution of lipid molecules.

Phospholipase A₂ (PLA₂), lysophospholipid acyltransferase, phospholipase D, SPT:

Brown et al. [21] emphasize the role of PLA₂ in inducing curvature producing an inverted cone-shaped lysophospholipid from a cone- or cylindrical-shaped phospholipid. The reverse reaction is catalysed by lysophospholipid acyltransferase. A role of the latter reaction is discussed at the neck of a vesicle before fission [22,23] and was challenged by Gallop et al. [24]: the rigorous purification of endophilin or CtBP/BARS (carboxy-terminal binding protein/brefeldin A-ribosylated substrate), proteins with a putative lysophospholipid acyltransferase activity, showed a too low an activity to effect curvature. Gallop et al. [24] see the role of endophilin in vesicle tethering rather than in changing curvature. They confirmed: "the dynamic modulation of membrane curvature by localized lysophospholipid acyltransferase activity is an attractive proposition" but the facts testify against this role. Nevertheless one should also consider that the activity especially of membrane enzymes could change a great deal dependent on the local environment. Thus one should not completely reject that under natural conditions lysophospholipid acyltransferase may play a role during vesicle formation/fission.

As mentioned before, lysophospholipid acyltransferase and PLA₂ catalyse reverse reactions. Meyer et al. [25] showed that fatty acids from various glycerophospholipids freed by PLA₂ were used for sphingolipid synthesis changing physical properties of the membrane. Besides PLA₂ a putative phospholipase D may play a role because [¹⁴C]serine from phosphatidyl [¹⁴C]serine is a substrate for sphingolipid synthesis [26]. In yeast a phospholipase D activity splitting phosphatidylethanolamine and phosphatidylserine was described as an unconventional phospholipase D activity dependent on Ca⁺⁺ and lacking transphosphatylation activity [27]. SPT is the first committed enzyme in sphingolipid synthesis and thought to reside in the endoplasmic reticulum. It normally uses free serine and the CoA-thioester of mostly palmitate. In recent years it has turned out that there are, in addition to the known subunits (LCB1 and LCB2), other proteins, ssSPTa and ssSPTb [28] or SPLC3 [29] influencing activity and/or substrate specificity of SPT. However sphingolipid metabolism is still not completely characterised. For example, LCB1, a subunit of SPT, usually thought to reside in the endoplasmic reticulum, was shown to be present in focal adhesions [30]. The discovery of a subunit of SPT in focal adhesion suggests that we may find further sphingolipid synthesis enzymes at the plasma membrane. They can easily escape discovery if they are only temporarily activated.

Sphingomyelinases: Another reaction is discussed by Zha et al. [31]. They describe an ATP-independent “endocytosis” by treatment of membranes of ATP-depleted macrophages and fibroblasts with exogenous sphingomyelinase. This enzyme induced vesiculation. They speculate that sphingomyelinase causes inward curvature. The participation of acid sphingomyelinase in CME is shown by A. Wendt (thesis in preparation, our laboratory): Desipramine, an acknowledged functional inhibitor of acid sphingomyelinase [32], reduces the uptake of transferrin in experiments executed as in [2] by about 60 %. After a stimulus acid sphingomyelinase is translocated to the outer leaflet of the plasma membrane degrading sphingomyelin there [11]. The inhibition of transferrin uptake by desipramine suggests a role of this sphingomyelinase in CME producing inward curvature with the formation of ceramide in the developing vesicle.

Sphingomyelin synthase: Sphingomyelin synthesis is thought to take place in the luminal leaflet of the trans Golgi network [33] Sphingomyelin is delivered in vesicles to the outer leaflet of the plasma membrane by the secretory pathway. The bulk of sphingomyelin is therefore found at the outer leaflet of the plasma membrane because sphingomyelin can neither flip to the other leaflet spontaneously, nor is a flippase that would catalyse its transfer to the inner leaflet known. Duran et al. [33] concluded from their research with C₆-sphingomyelin that sphingomyelin synthesis is responsible for liquid-ordered sphingomyelin-rich domains at the Golgi membranes and essential for transport carrier (vesicle) formation. Sphingomyelin synthesis at the plasma membrane by sphingomyelin synthase (SMS2) was reported by [34], though it is assumed that this synthesis takes place on the outer leaflet of the plasma membrane. In the proposed glycerophospholipid cycle (Figure 1) sphingomyelin synthesis is speculated to take place at the inner leaflet of the plasma membrane. It is beyond controversy that at least a small pool of sphingomyelin is at the inner leaflet of the plasma membrane even though the origin of this sphingomyelin pool is not known. Linardic et al. [35] using HL-60 and U937 human leukemia cells found two distinct sphingomyelin pools, one at the outer and one at the inner leaflet of the plasma membrane. Also Andrieu-Abadie [36] measured resynthesis of sphingomyelin after TNF- α stimulation on the cytosolic leaflet under conditions where the traffic and endocytosis (4 °C) is blocked. Sphingomyelinase from outside did not change this sphingomyelin pool. They concluded that there is a pool of sphingomyelin

at the inner plasma membrane that is also synthesized there. Neutral sphingomyelinase is an important player in signalling. This enzyme is located at the inner leaflet of the plasma membrane as predicted by [37]. Without the presence of sphingomyelin at the inner leaflet this enzyme would have no substrate. Van Blitterswijk et al. [15] suggest a transfer of sphingomyelin from the outer leaflet to the inner leaflet due to phospholipid scrambling in the effector phase of apoptosis whereas during the initiator phase of apoptosis the neutral sphingomyelinase would produce ceramide from the minor sphingomyelin pool at the inner leaflet.

Summarizing the discussion it is a fact that glycerol- and sphingophospholipid-metabolizing enzymes are connected to severe shape changes of the lipid molecules and thus induce changes in curvature. Compartmentalisation of these enzymes in, at and to the plasma membrane is a prerequisite for their role in forming a vesicle at the plasma membrane.

The hypothetical glycerophospholipid cycle (Fig. 1)

Ceramide may be de novo synthesized by a special serine palmitoyltransferase located at the inner leaflet of the plasma membrane using serine from phosphatidylserine and a fatty acid from for example phosphatidylcholine (or glycerophospholipids with other head groups) via dihydrosphingosine (DHS). Sphingomyelin is synthesized from this ceramide then at the inner leaflet of the plasma membrane. It increases the number of molecules of the inner leaflet of the plasma membrane and it contributes to curvature in direction of the cytosol. Contrary to the fact that a flippase (transport of lipids from the outer leaflet to the inner leaflet) for sphingomyelin is unknown, sphingomyelin could be flopped from the inner leaflet to the outer leaflet by one of a spectrum of ATP-binding cassette (ABC) transporters [38]. At the outer leaflet acid sphingomyelinase can produce ceramide from sphingomyelin, further promoting the needed curvature for forming a vesicle. Ceramide induces negative curvature and may now become the centre of the new signalosome [11] of the developing vesicle. Later ceramide might be degraded by a ceramidase. A role of this enzyme was shown in R1-R6 photoreceptor cells of *Drosophila* facilitating membrane turnover and endocytosis of the G protein receptor Rh1 [39]. Sphingosine (product of ceramidase) can be phosphorylated by a sphingosine-

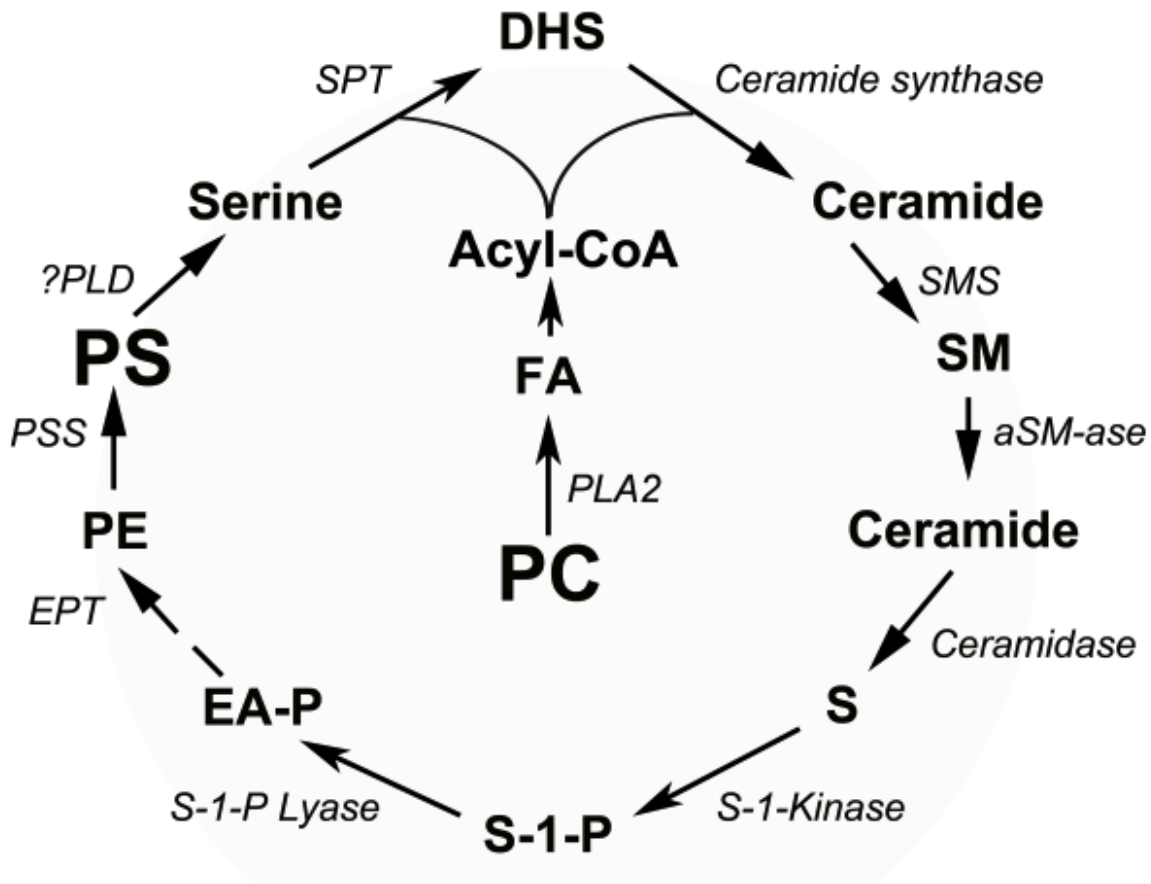


Figure 1 - Hypothetical glycerophospholipid cycle. For explanation see text.

Abbreviations: 1. **Substrates:** PS phosphatidylserine, DHS D-erythro-dihydrospingosine (sphinganine), SM sphingomyelin, SPH sphingosine, S-1-P sphingosine-1-phosphate, EA-P ethanolamine phosphate, PE phosphatidylethanolamine, PC phosphatidylcholine, FA fatty acid; 2. **Enzymes:** PLD phospholipase D, SPT serine palmitoyltransferase, SMS sphingomyelin synthase, aSM-ase acid sphingomyelinase, S-1-Kinase sphingosine-1-kinase, EPT CDP-ethanolamine: 1,2-diacylglycerolethanolamine phosphotransferase, PSS phosphatidylserine synthase 2; assembled from [2,25,26,47,48,49,50].

1-kinase to sphingosine-1-phosphate, a signalling molecule with predominantly growth promoting properties. Sphingosine-1-phosphate lyase can break sphingosine-1-phosphate into phosphoethanolamine and hexadecenal. Phosphoethanolamine can end up in phosphatidylethanolamine [40] a precursor of phosphatidylserine to close the cycle. The sequential synthesis and breakdown of the metabolites of the suggested glycerophospholipid cycle with the distinct physical properties of each metabolite may at least partly (besides curvature influencing proteins) participate in forming a vesicle.

Many substrates in the proposed glycerophospholipid cycle are known signalling molecules. DHS and phytoshingosine (sphingoid

bases) are known to activate PKH1/2 kinases (homologous to mammalian 3-phosphoinositol-dependent kinase-1 (PDK1)), which are able to suppress the requirement of sphingolipids in endocytosis in yeast [41]. PKC1 is a downstream kinase of PKH1 involved in endocytosis [42]. Meyer et al. [2] showed comparable results in L929 cells: Transferrin uptake inhibition by myriocin could be lifted by 4 β -phorbol-12-myristate-13-acetate (PMA) (an activator of PKC) and by okadaic acid, an established inhibitor of serine/threonine protein phosphatases [43,44]. Ceramide is a widely known signalling molecule in growth arrest, cell differentiation, apoptosis and also in growth (see introduction). It is direct an activator of an atypical

protein kinase ζ and together with ceramide-dependent protein phosphatase 2A it plays a role in, for example, insulin signalling [45]. Sphingomyelin synthesis by sphingomyelin synthase 1 and thus sphingomyelin is needed for transferrin uptake and cell proliferation in lymphoma cells [3] though the molecular mechanism is unknown. Cheng [1] investigating mutated Chinese hamster ovary cells showed that sphingolipid depletion induced disruption of RhoA and Cdc42-regulated endocytosis, which could be partly restored by sphingomyelin probably targeting RhoA and Cdc42 to the plasma membrane. Here the problem of compartmentalization stays unresolved (sphingomyelin must be on the inner leaflet for targeting tasks). Sphingosine-1-phosphate is a bioactive sphingolipid involved in many cell processes [46]. The breakdown of sphingosine-1-phosphate ends up in phosphatidylethanolamine. In L929 cells phosphatidylethanolamine increased at the expense of sphingomyelin as an answer to TNF- α and staurosporine (Meyer, unpublished).

In order to examine this suggested hypothesis the search for the enzyme proteins in the coated pits or vesicles is more promising than the measuring of enzyme activity taking into consideration the probably only temporary activation of the involved enzymes.

Summarizing the discussion we propose that the endocytosis starts from glycerophospholipids, phosphatidylserine (and other glycerophospholipids) as substrates for sphingolipid synthesis. The following breakdown of sphingolipids serves to restore phosphatidylserine. Each of the intermediate of the cycle may be a signalling molecule (activating, for example, the next step and deactivating the step before) and a cause for the change in the lipid structure of the membrane.

My hypothesis says that in addition to known curvature-inducing and sensing proteins synthesis and breakdown of lipids especially sphingolipids (represented in the proposed glycerophospholipid cycle) play a decisive role in domain and vesicle formation during CME.

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