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Review

How lipid flippases can modulate membrane structure

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ABSTRACT

Phospholipid flippases, are proteins able to translocate phospholipids from one side of a membrane to the other even against a gradient of concentration and thereby able to establish, or annihilate, a transmembrane asymmetrical lipid distribution. This lipid shuttling forms new membrane structures, in particular vesicles, which are associated with diverse physiological functions in eukaryotic cells such as lipid and protein traffic via vesicles between organelles or towards the plasma membrane, and the stimulation of fluid phase endocytosis. The transfer of lipids is also responsible for the triggering of membrane associated events such as blood coagulation, the recognition and elimination of apoptotic or aged cells, and the regulation of phosphatidylserine dependent enzymes. Exposure of new lipid-head groups on a membrane leaflet by rapid flip-flop can serve as a specific signal and, upon recognition, can be the cause of physiological modifications. Membrane bending is one of the mechanisms by which such activities can be triggered. We show that the lateral membrane tension is an important physical factor for the regulation of the size of the membrane invaginations. Finally, we suggest in this review that this diversity of functions benefits from the diversity of the lipids existing in a cell and the ability of proteins to recognize specific messenger molecules.

Contents

1. Introduction: what do flippases do?

One particular feature of living organisms (eukaryotes as well as prokaryotes) is that the association of molecules that form such systems is not in a thermodynamic equilibrium. There is continuous renewal of molecules, involving a sophisticated traffic controlled by messenger molecules and/or by modulation of physical properties. The turnover of biomolecules depends considerably on the type of molecule. Therefore, protein activity has to be continuously regulated to guarantee a fragile steady state situation. Investigation of model systems such as pure lipid vesicles constitutes a useful approach, but can be misleading in that regard. Although lipids form symmetrical and stable bilayers in artificial liposomes with a random spontaneous transbilayer lipid diffusion (or flip-flop) between both leaflets, lipids in cell membranes are in a metastable asymmetrical lipid organization, maintained by lipid transporters. The maintenance of an asymmetrical lipid repartition between the two sides of biomembranes by lipid pumps called flippases is a typical example of an out of equilibrium situation in biology requiring energy consumption. The transbilayer diffusion of phospholipids is generally a slow event, associated with

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very long residence time of lipids in each monolayer (several hours for long chain phospholipids [1,2]). Nevertheless, the plasma membrane composition would eventually be randomized in the absence of selective flippases able to correct lipid redistribution mediated by the transbilayer lipid diffusion. In eukaryotes, membrane lipids are renewed via exocytosis–endocytosis traffic, which permits exchange of lipids between the plasma membrane and the inner membranes where lipid synthesis occurs, principally in the ER and the Golgi apparatus. In erythrocytes, where very little lipid metabolism takes place, and no intracellular membranes are present, new lipids are provided essentially by serum lipoproteins of the blood. Eventually the asymmetrical distribution of lipids in eukaryotic membranes is established by ATP dependent selective lipid translocators or flippases. Several different proteins are responsible for the establishment and maintenance of lipid asymmetry in eukaryotes, or for non-specific lipid redistribution (called "scrambling") (for reviews see [2–10]). The ensemble of those proteins might be considered as a functional module, whose task is to regulate the lipid asymmetry [11–13].

Discovered functionally in the plasma membrane of human erythrocytes in 1984 [14], ATP dependent flippases are ubiquitous proteins in the plasma membrane of eukaryotes. Similar lipid translocators may exist in specialized organelles (Golgi) [15]. Lipid translocators have also been reported in prokaryotes. The latter belong to the so-called *A*TP *B*inding *C*assette family of ATPases, which are actually present in both pro- and eukaryotic cells [16–19]. The most representative lipid translocator of this ABC family is the P-glycoprotein (ABCB1) responsible for multidrug resistance [16].

The term *flippase* is often used abusively as a generic term to designate a lipid translocator, which catalyzes the passage of lipids from one leaflet to another but it is misleading because different functions are involved. The transmembrane passage of lipids in biomembranes is facilitated by *three* types of proteins: *flippases*, *floppases* and *scramblases*. They are functionally differentiated in Fig. 1. Further subdivisions should be made since *flippases* can be ATP dependent or ATP independent, they can be selective or non-selective. Note that the expression "*lipid transporter*" should preferentially be avoided to prevent confusion with the water soluble proteins that can shuttle lipids from one membrane to another. According to the nomenclature used frequently, the ATP dependent "flippase" (also called aminophospholipid translocase) transports aminophospholipids (PS and PE) in eukaryotic cells from the plasma membrane outer monolayer to the inner monolayer. However recent data showed that even phosphatidylcholine (PC) can be transported by an ATP dependent flippase in mammalian cells [20] and yeast [21]. The P-glycoprotein, which should be named a *floppase* transports from the inner to the outer monolayer, at the expense of ATP hydrolysis, amphiphilic drugs and phospholipids with little selectivity [16–19]. Notably, transport of phospholipids was concluded essentially from experiments using fluorescent short chain analogues. However, a recent study suggests that P-glycoprotein selectively translocates such analogues but not natural long chain lipids [22].

Flippases' identifications, localization and purification have been discussed in many former reviews [see above]. P-type ATPases were recognized as the most likely candidates for inward lipid translocation in eukaryotic membranes (flippase) [22–25]. However, it is important to acknowledge that in spite of extensive work carried out in several independent laboratories, none of the putative flippase proteins, with the exception of the P-glycoprotein, which is a floppase, have been purified yet in large enough quantity to allow one to carry out the necessary experiments to measure in proteo-liposomes the efficiency and specificities of these ATPases nor is there hope to achieve rapidly successful crystallization for structure determination. The best strategy so far to identify the translocases and their function has been to use knock-out cells or natural mutants depleted of specific ATPases which were suspected to be candidate flippases. Molecular biology approaches have revealed defects in lipid transport in yeast mutants and in other systems including plant cells, where particular ATPases were absent [15,21,21,22–26]. Various phenotypes were observed with these mutants, for example loss of chilling tolerance of plant cells [24], while endocytic activity was abolished in yeast mutants with triple knock outs of P-type ATPases [21]. See below for more details. Budding of yeast Golgi membrane could also be associated with a P-type ATPase [26]. Such observations are very important to find out whether ATP dependent flippases are able to generate membrane bending *in vivo*.

The scramblase flips aminophospholipids in particular PS from the plasma membrane inner to outer monolayer upon increased level of calcium in the cytosol [27]. The exposure of PS on the outer leaflet is necessary for platelet aggregation because PS is a cofactor for the conversion of prothrombin into thrombin, which is followed by the formation of fibrin strands and of the clot that stops accidental bleeding [28]. Remarkably, scramblase activity is present in red blood cells (RBCs) although its biological function in these cells is not known. The scramblase activity is supposed to be caused by an ATP independent transmembrane protein, still unknown at a molecular level, which would be triggered by the presence of cytosolic calcium [27–29]. The scramblase facilitates the flip-flop of lipids in a non-selective fashion. In the presence of calcium, the scramblase behaves like a channel for lipids allowing them to diffuse from one monolayer to the other according solely to the concentration gradient. The involvement of a 37 kDa protein had been reported but later the function of this protein as a lipid translocator has been challenged (see [30]). We have actually proposed recently that a calcium dependent soluble SMase can trigger scrambling of lipids by destabilizing the plasma membrane via conversion of the inner leaflet sphingomyelin to ceramide, a lipid with a very small polar head group. The change in area occupied by the new lipid in one leaflet can form temporary pores along which lipid flip-flop would be facilitated [31] (see below).

The present review focuses on flippase activities that modulate the *structure* of membranes by redistributing lipids between the two leaflets. The term *structure* here does not refer directly to the 3D-structure of proteins or of lipids. Except in specific cases, a change of structure should be understood here at macroscopic level, meaning first a change of membrane shape created by local or non-local membrane bending. A change of structure can also mean a new organization of lateral or transmembrane lipid domains. In general, a change in the distribution of lipids between the two monolayers acts

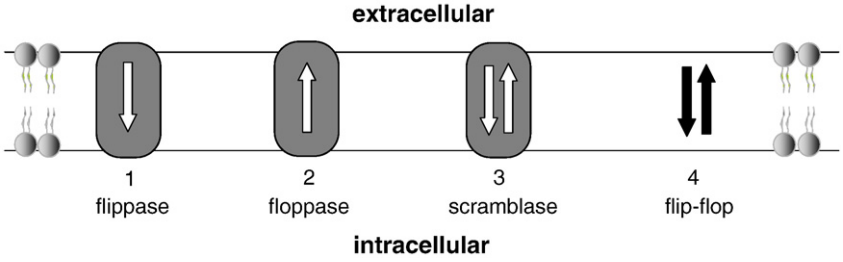

Fig. 1. Traditional definition of the various proteins involved in lipid translocation within biological membranes.

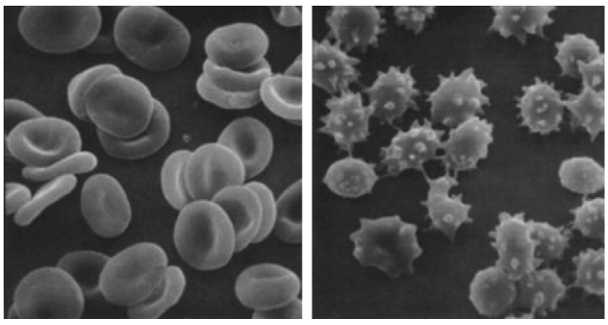

Fig. 2. Shapes of human erythrocytes as seen by scanning electron microscopy: left, normal fresh erythrocytes; right, after 30 h of metabolic depletion. Shapes identical to those seen in the right panel are obtained instantaneously if lyso-phosphatidylcholine or phosphatidylcholine with short chains is added to fresh erythrocytes. Addition of phosphatidylserine or phosphatidylethenolamine induces also the formation of spicules but only temporarily. Figures from Ferrell and Huestis [42].

as a switch for different biological functions. In the case of apoptosis, it is a signal of cell death, which is used for cell phagocytosis by macrophages. Furthermore, alteration of the concentration of the negatively charged PS on the inner plasma membrane leaflet reduces the electrostatic attraction to proteins with basic cluster, leading to a redistribution of those proteins from the membrane to the cytosol by a simple 'electrostatic switch' mechanism [32,33].

2. How does lipid asymmetry trigger membrane bending?

Perhaps the most spectacular effect of lipid translocation from one monolayer to the other of a cell membrane is the shape change which can be either local or involve the whole cell. Very early, it was shown by Sheetz and Singer that the insertion of a small fraction (~1%) of exogenous lyso-PC or of chlorpromazine in RBCs is accompanied by spectacular shape changes that were rationalized by the authors in the famous *bilayer couple model* [34]. This model, initially rather intuitive was progressively refined and extended by theoreticians who eventually were able to account for pure lipid vesicle shapes with simulations based on minimization of bending energy [35–41]. The experiments carried out by Ferrell and Huestis in 1984 (see Fig. 2 and [42]) where they showed that the conversion of phosphatidylinositol (4,5) bis phosphate (PIP2) to phosphatidylinositol (a molecule that has a smaller polar head group) coincides with the shift from discoid cells to the crenated ones may be viewed in the framework of the Sheetz and Singer model. The metabolic crenation arises from a loss of inner monolayer area secondary to the degradation of PIP2. However, this shape change is a general feature of RBCs and can take place spontaneously in case of metabolic depletion. It reveals that the steady state shape (discoid conformation) is a non-stable structure, requiring a continuous adjustment of the asymmetric transbilayer lipid distribution *in vivo* which is perturbed due to the slow but non-neglectable diffusion of aminophospholipids towards the outer monolayer [43].

As demonstrated first with lipid monolayers in Langmuir–Blodgett troughs, lipids in a condensed lamellar state are very difficult to compress laterally, a consequence of which being that the transmembrane diffusion of lipids in a bilayer is an unlikely event. In other words, the slow flip-flop of lipids is not solely due to the difficulty of polar moieties to penetrate the lipid bilayer. The addition of new lipids in one leaflet of a membrane can be performed if the new lipid comes from the aqueous phase where it is in an unfavorable environment. Lipids, with a low solubility in water, for example lyso-PC with a long chain or a phospholipid with two relatively short chains, insert into a membrane or a micelle because such amphiphiles prefer the hydrophobic environment constituted by a membrane or a micelle. However, to flip from a hydrophobic environment to a second identical hydrophobic environment that is to go from one leaflet to the other by a flip-flop process does not save any energy. On the contrary, the monolayer lateral pressure caused by the insertion into the second monolayer can produce an energy barrier for flip-flop, which may be over k_BT even for lipids with a small head group. Bending a lipid bilayer consumes much less energy per inserted lipid molecule. A consequence is that the insertion of even a small proportion of lyso-PC into a Giant Unilamellar vesicle (GUV) is accompanied by the formation of a single bud [44–47]. In the case of biological membranes, Sheetz and Singer in 1974 showed that the insertion of lyso-PC into the red cell membrane triggered a membrane shape change with the formation of echinocytes characterized by the presence of many spicules. Sune and Bienvenüe in 1988 showed that platelets give rise to a very different shape characterized by several long pseudopods (Fig. 3 and [48]). The different response between GUVs, erythrocytes and platelets can be rationalized only if one takes into account the existence of a cytoskeleton in biological membranes. Within platelets an important modification of the cytoskeleton involving actin polymerization is triggered by the modification of the lipid distribution. An unexpected feature of this

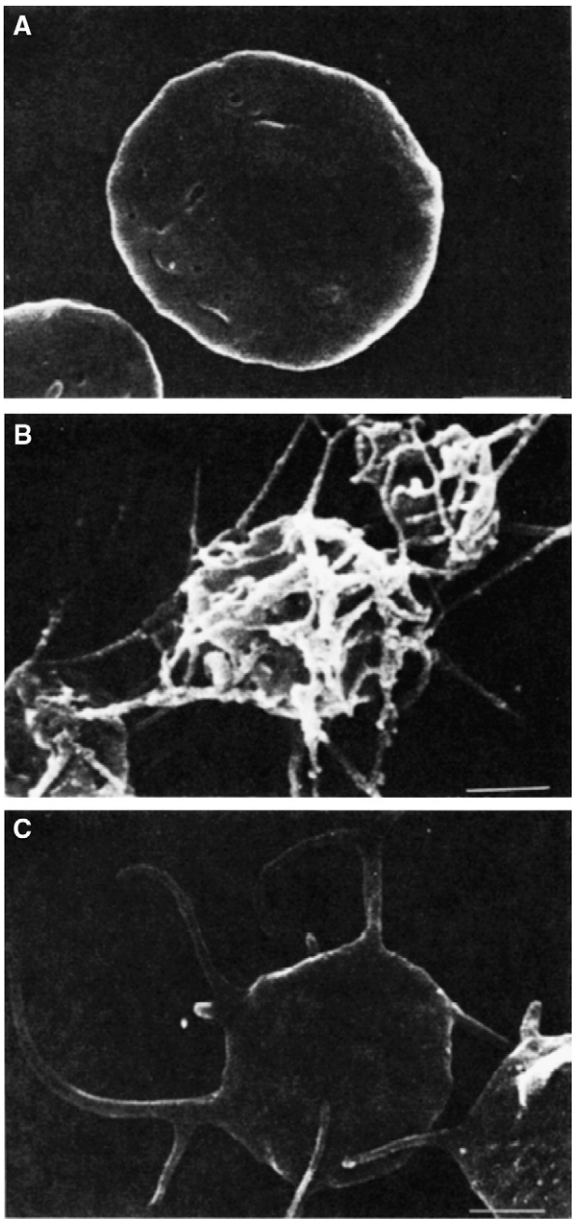

Fig. 3. Shapes of platelets after incubation with spin-labeled phosphatidylethanolamine (from Sune and Bienvenue [48]).

system is that, apparently, a chemical modification and not only a change of conformation of actin is triggered by a physical constraint imposed by the bilayer modification, which comes from an increase of the bilayer lateral tension. This shows that membrane shape changes cannot always be deduced from minimization of the energy of the lipid bilayer alone, but that the effects of the intracellular structures if they exist should be taken into account.

There are actually several ways by which membrane bending can be generated. The mere resealing of small bilayer sheets to avoid exposure of hydrophobic alkyl chains to the water is the basis of the formation of Small Unilamellar Vesicles by sonication (SUVs). The diameter of SUVs is probably determined by the maximum bending of a lipid bilayer. McMahon and Gallop in 2005 [49] and Zimmerberg and Kozlov in 2006 [50] have indicated several phenomena less perturbing than sonication that can concur to membrane bending. One mechanism which is largely emphasized in the literature (perhaps overemphasized!) is the influence of so-called spontaneous curvature of individual lipids. It is true that a mixture of PE and PC when sonicated forms asymmetrical SUVs with most PE in the inner monolayer and PC in the outer monolayer [51]. However many speculations, yet to be confirmed by experiments, were made about the role of lipids in shape generation of organelles with a high curvature, for example disks in rod outer segments or mitochondrial invaginations, or Golgi. It has been suggested that the packing of such organelles requires special lipids to accommodate the high curvature observed in such membranes. However, a serious ambiguity remains if the spontaneous curvature of specific lipids is associated with a biologically significant modulation. For example during endocytosis: do lipids induce a particular bilayer curvature (as often suggested) or do the lipids diffuse towards a region of a membrane where they find the curvature best adapted to their effective molecular shape? If the latter hypothesis would be true, one could not explain why shape changes happen since the bending ought necessarily to be established *before* the recruitment of new lipids! Thus, the origin of the curvature would not be explained by the lipid properties in this model, nor of course could the model explain the requirement of ATP hydrolysis, for example in the case of budding during endocytosis. Finally if one considers the size of the regions of "high curvature" generally associated with an image obtained by optical microscopy (hence with a resolution of about 1 μm) it should be admitted that the "high curvature" is not so high at the scale of lipids. Summarizing the phenomenology of the role of lipids in the membrane shape generation, it can be concluded that because of the effective incompressibility of the lipid monolayers the bilayer spontaneous curvature is largely determined by the monolayer area difference, $A^{out}-A^{in}$, and in case the latter does not come into play, $A^{out}=A^{in}$, the difference in the monolayer spontaneous curvature becomes relevant [50].

It seems clearly established that an ATP dependent specific flippase is a protein that modifies the monolayer area difference and uses ATP for this purpose. Other proteins that could play a role in membrane bending (but do not need ATP), are proteins from the cytoplasm that interact spontaneously with one monolayer and generate membrane bending. The examples are the membrane scaffolding by the BAR domain containing proteins, and by the complexes of clathrin, adaptor proteins and their membrane receptor [50] or the insertion of epsin into one of the membrane monolayers [52]. All these proteins created an asymmetrical membrane because the proteins that transform the membrane are initially only present on the cytoplasmic side of the plasma membrane.

3. Lipid asymmetry and membrane bending: the particular case of RBCs

Flippase activity was suspected initially in RBCs from the data published by M. Bretscher in 1972 [53] on the transmembrane orientation of phospholipids in human erythrocytes. Subsequently, Bretscher coined in a review in 1973 the word "flippase" [54]. The existence of such a protein in human erythrocytes and in other blood cells (platelets and lymphocytes), as well as its Mg-ATP requirement was clearly demonstrated [14,48,55]. Other papers by various groups showed the ubiquitous character of the flippase in eukaryotic cells. Already in the paper in 1984, the importance of the flippase activity on the shape of human erythrocytes was emphasized. This aspect was further investigated in detail by Daleke and Huestis in 1985 [56]. However, in erythrocytes the shape deformation is not a simple local invagination. Erythrocytes have normally a characteristic discocyte shape, which optimizes the cell capacities to form aggregates called "rouleaux" (in which cells resemble "coins in a stack" (Fig. 4 and [57])). Rouleaux formation is a way to optimize the blood flow in microcapillary hemodynamics. Rouleaux also provide the maximum surface-to-volume ratio necessary for effective exchange of oxygen. Actually, all abnormal red cell shapes as echinocytes, stomatocytes, spherocytes, and sickle cells correspond to red cell diseases which are associated with impaired rheological capacities [58]. The discocyte shape is not an equilibrium situation. If blood is stored too long in a blood bank (several weeks) it is progressively depleted of ATP and the flippase is unable to maintain the discocyte shape: echinocytes or spherocytes are generated. Furthermore, for erythrocyte with an insufficient aminophospholipid translocase activity, PS cannot be maintained in the inner monolayer and accidental clotting can happen and cause thrombosis.

As discussed briefly above, cell shapes (and liposome shapes) are at least partially determined by the spontaneous bending of the lipid bilayer due primarily to the monolayer area difference $A^{in}-A^{out}$. The cytoskeleton is the other component of eukaryotic cells involved in shape determination. Thus, the lipid transmembrane distribution plays a crucial role in the determination of erythrocytes shapes. However, in the absence of cytoskeleton, vesicle shape deformation following the asymmetrical addition of lipids does not mimic the erythrocyte shapes sequence [60]. Thus the mere calculation of liposome shapes by minimization of bending energy [61] is not sufficient to properly simulate red cell shapes unless new *ad hoc* hypotheses are introduced to account for the cytoskeleton effects [41,43]. Another argument in favor of the cytoskeleton importance comes from the difference between the various shapes of the blood cells. In spite of the fact that the lipid compositions are very similar and that all blood cells possess the aminophospholipid translocase, their shapes are very different. Furthermore, modifications of the lipid transmembrane distribution, caused by ATP depletion, result in further dramatic differences in shapes. The deficiency of ATP content is associated with the failure of the flippase to keep aminophospholipids on the inner monolayer. Actually, any defect in flippase function can lead to a modification of cell structure, which hampers their rheological property. In addition the exposure of PS on the outer monolayer is a direct

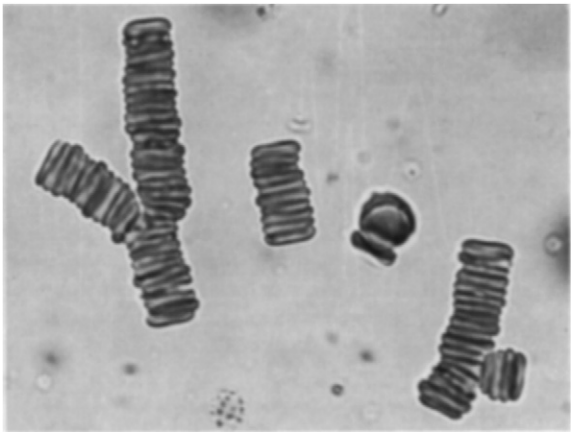

Fig. 4. Rouleaux of human erythrocytes (from Samsel and Perelson [57]).

signal to indicate to the macrophages the improper structure and function of the red cell.

The shape change of human erythrocytes after insertion of a small percentage of exogenous amphiphiles such as lyso-PC or chlorpromazine is different from what is observed when the same molecules are introduced into either GUVs or platelets. Clearly, echinocyte formation is associated with the presence of the cytoskeleton, which imposes certain boundary conditions to the buds. It is therefore likely that the cup shape corresponding to stomatocytes is also a result of a combined action of the bilayer and cytoskeleton interaction. Possibly, the absence of small invaginations in erythrocytes may be due to insufficient lateral tension of the membrane, which seems to be required to generate invaginations with a high curvature (see below, section 7).

The very high sensitivity of GUV shape to a small excess of lipids in one monolayer provides an interesting way to study flip-flop of unlabeled lipids. Indeed when a small amount of lipids is inserted into the external leaflet of GUVs, before randomization the asymmetric lipid supply, a surface area difference is created between the two leaflets, which in turn results in formation of a bud-like structure [45–47]. When added lipids and/or lipids from GUVs are allowed to redistribute between the two leaflets, the bud is unstable, and the original shape is recovered [44,45]. This can be explained by a relaxation of the monolayer area difference due to flip-flop. Otherwise, in the absence of flipping lipids, the bud remains. The time dependence of shape changes can be used for derivation of the flip-flop rate constant. We have recently reconstituted the energy-independent flippase activity from yeast endoplasmic reticulum into GUVs and have shown that the time course of GUV shape changes can serve also as an approach for quantitative characterization of the lipid transport activity of a flippase [47]. This approach should also allow investigation of energy-dependent lipid transporters. Although transporters are very likely reconstituted to a similar extent in both opposing directions, unidirectional lipid transport can be ensured by allowing ATP to access only one membrane leaflet. Notably, lipid species to be transported can already be incorporated during GUV assembly. This is different from the visualization of ATP independent flippase activity in GUVs where shape changes have to be triggered first, *e.g.* by supplementing the external leaflet with additional lipids.

4. Lipid asymmetry and vesiculation

As suggested already in 1991 [3], membrane invaginations that result from the transport of phospholipids from the outer to the inner monolayer by the aminophospholipid translocase are reminiscent of the endocytic process in eukaryotes. In 1994, Müller et al. showed that addition of PS to ATP containing erythrocyte ghosts stimulates the formation of the so-called endocytic vesiculation while PC (which is not transported by the flippase in red cells) had the opposite effect [62]. In principle, other flippases with a different selectivity for phospholipids to be transported could account for monolayer area difference. However, there is no report of membrane budding created by the P-glycoprotein, which confirms that the lipid transport by ABC protein is less efficient than that due to P-type ATPases.

Erythrocytes are of course an improper system to investigate endocytosis since there is normally no endocytosis in erythrocytes, only the formation of a single *large* invagination, which does not shed from the plasma membrane for low levels of ATP (~2 mM) [59]. For 10 mM ATP in the cytosol, small inside-out vesicles are formed. They can be separated from erythrocyte ghosts through a hypodermic needle. Fragmentation leads to a full breakdown of the ghosts into small vesicles. Such ATP induced vesicles do not seem to have a physiological function [58].

K562 cells are transformed cells derived from human erythroblasts, a precursor of erythrocyte. These cells have an efficient endocytic activity [63]. Farge and collaborators used K562 cells to test the possible stimulation of endocytosis by adding exogenous aminophospholipids to the outer monolayer of K562 plasma membrane [64,65]. PS or PE with a long α-chain and a short β-chain were used in order to permit an easy membrane incorporation of the exogenous lipids. Such lipids, which were added directly to the buffer containing the cells, have enough water solubility to allow a direct addition to the cell suspension without the need of a phospholipid exchange protein.

Fig. 5 shows endocytosis stimulation by PS and PE as reported by Farge et al. and the partial inhibition of endocytosis due to the addition of lyso-PC to the cell surface: lyso-PC is not transported by the translocase in K562 cells and has a very different effect on the rate of endocytosis [65]. More recently, the role of the putative lipid transporters of the Drs2p family of ATPases in the formation of lipid vesicles was illustrated by two different groups. In 2004, T. Graham showed the requirement for yeast Drs2p family of P-type ATPase in both phospholipid translocation and protein transport in the secretory and endocytic pathways [9]. As pointed out by Graham several lines of evidence indicate that phospholipid translocation by Drs2p is required for vesicle formation. Inactivation by temperature of drs2-ts, a temperature sensitive mutant, rapidly inactivated NBD-PS translocation *in vitro* and dense vesicles formation *in vivo*, indicating that the ability of Drs2p to flip PS to the cytosolic leaflet of the trans-Golgi Network is required to support vesicle formation. In 2006, Alder-Baerens et al. [66] showed that loss of Drs2p and of Dnf3p (yet another member of the P4-ATPases) disrupts aminophospholipid transport and asymmetry in yeast post-Golgi secretory vesicles. This finding confirms the role of the lipid flippase in the formation of vesicles.

Finally it is important to mention here the observation by Roux et al. who have shown that under particular conditions a change of

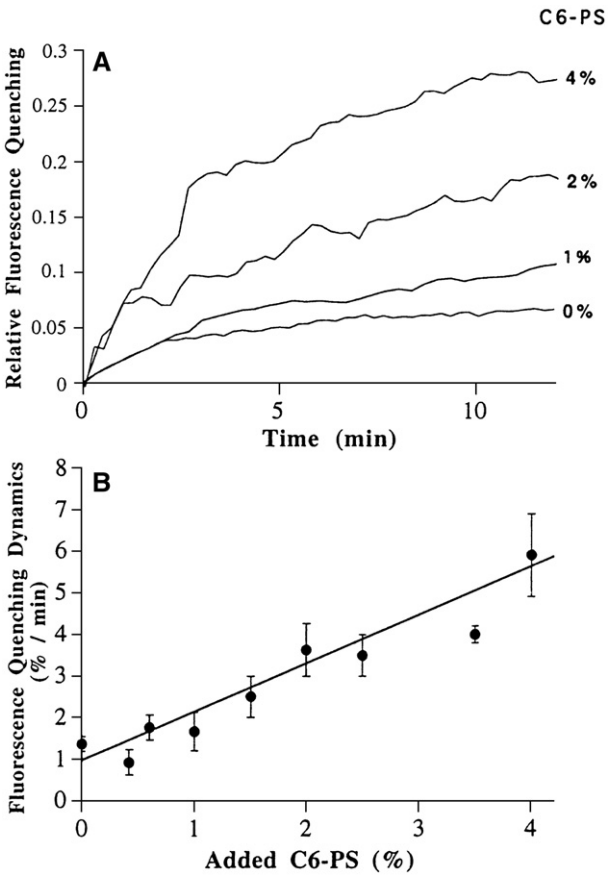

Fig. 5. Stimulation of endocytosis in K562 cells due to the addition of phosphatidylserine to the outer leaflet of the plasma membrane. Endocytosis was measured by the decrease of fluorescence intensity of labeled proteins from the plasma membrane, hence fluorescence quenching is here a measure of endocytosis activity (from Farge et al. [65]).

membrane structure can give rise to tubules instead of vesicles [67], the lipid composition in the tubules was significantly different from the composition of the vesicles, furthermore fission was observed when phase separation was generated in the tubes. The authors concluded that lipid sorting must depend critically on both the membrane curvature and phase separation.

5. Lipid asymmetry and monolayer tension

The following paragraphs deal with *in situ* enzymatic modifications of membranes, which can cause significant structure modifications by modifying the lipid transmembrane asymmetry in such a way that the bilayer cannot adjust to a sudden surface area differences between the two monolayers without the formation of transitory pores. This specific topic is based on observations made by several laboratories but has not yet led to a consensual interpretation. The initial observation is the following: if a membrane (vesicle or biomembrane) contains sphingomyelin and if exogenous SMase is allowed to transform SM into ceramide by removing the head group of SM on one side of the membrane, the modification is associated with a partial scrambling of *all phospholipids*. Goñi's researchers who observed this phenomenon first, have attributed it to a direct influence of ceramide on the other lipids possibly due to the formation of non-bilayer structures [68]. However in a recent article [31] we have shown that in liposomes containing as low as 5% SM, enzymatic attack by SMase triggers a drastic perturbation which provokes the formation of a large transitory pore, visible by optical microscopy and by AFM. Although further studies are required, we hypothesized that the generation of a pore can be explained by the mismatch between the two monolayers after conversion of SM into ceramide. The latter molecules have a head group much smaller than the former, thus the outer monolayer shrinks. However, because SM molecules in the inner monolayer do not flip, a severe mismatch is generated between the two monolayers, which creates an asymmetrical surface area, and, hence, a difference of lateral tensions between the two monolayers. The result is membrane bending with the formation of an invagination and in some cases of a pore. Even if 50% of the newly formed ceramide flips rapidly to the inner leaflet [30] there will still be a considerable mismatch. In a former article in 1992, we showed that along the edges of a pore, phospholipids can diffuse from one side of the membrane to the other. In the 1992 study, pores were formed in erythrocytes by hypotonic hemolysis [69].

6. Can the lipid flippase trigger structural changes of membrane proteins by the modulation of the lateral tension?

A new idea which is presently under study is the following: since the transport of lipids from one monolayer to the other by an ATP dependent flippase creates a lateral tension via the mismatch between the two monolayers, it is conceivable that this lateral tension is sufficient to modify the conformation of specific membrane proteins. To validate this hypothesis we have used GUVs containing a purified mechano-sensitive channel from *Escherichia coli* (provided by A Ghazi, Orsay, Fr). The flippase (non purified) was obtained from membrane fragments of human erythrocytes depleted of cytoskeleton and fused with the giant vesicles. Preliminary experiments indicated that the pressure necessary to open the channels with a micropipette seemed to be lowered when ATP was added. One explanation could be a contribution of the flippase to the opening of the *E. coli* channel. However, at this stage, we cannot exclude the role of other ATPases (P. Ezanno, A. Ghazi, P.F. Devaux unpublished). The success of these experiments, which need further investigation, will permit us to show that the flippase can modify not only membrane shape but also the conformation of membrane proteins, by the generation of lateral tension created by the transfer of lipids from one monolayer to the other.

7. Theoretical modeling of membrane bending by the monolayer area asymmetry in conjunction with the bilayer lateral tension

As mentioned above, membrane bending by the monolayer area asymmetry has been a subject of a thorough theoretical analysis [35–41]. These articles addressed, in some cases by sophisticated numerical approaches, shape transformations of closed membranes of vesicles with fixed volume and fixed membrane surface area. Such studies are helpful for understanding the experimental results obtained in artificial systems consisting of unilamellar vesicles whose membranes are subject to controlled generation of the monolayer area asymmetry. At the same time, treatment of membrane shaping in cells often requires consideration of other thermodynamic conditions for membrane behavior. Namely, in many biologically relevant situations, a cell preserves the lateral tension of its membrane rather than the intracellular volume (see for review [70,71]). While, generally, the theoretical predictions for membrane shape transformation under constant lateral tension can be derived from the results obtained for the conditions of constant volume, in practice this requires complicated numerical treatment, and appears unfeasible. For the sake of the present review, we undertook a direct theoretical consideration of the qualitative features of membrane shaping by the monolayer area asymmetry under fixed lateral tension.

We consider a closed membrane subjected to lateral tension γ and characterized by the mid surface area A. The spontaneous areas of the outer and inner membrane monolayers corresponding to their unstressed states are, respectively,

$$A_S^{out} = A_0 - \Delta A, \quad \text{and} \quad A_S^{in} = A_0 + \Delta A. \tag{1}$$

Here and below, the superscript "*out*" and "*in*" denote the outer and inner monolayer, respectively. According to the bilayer couple mechanism, the difference in the monolayer spontaneous areas, $2\cdot\Delta A$, generates curvature J of the membrane mid plane, which partially prevents accumulation of the monolayer stretching–compression energy. Note that A_S^{out} is assumed to be smaller than A_S^{in} which generates budding towards cytosol of a cell or inside volume of a liposome (Fig. 6). Our aim is to address the membrane configurations generated by the monolayer area asymmetry $2\cdot\Delta A$ upon a lateral tension γ.

According to the results of our analysis, whose details are presented in the Appendix, there are three possible regimes of the system behavior. Boundaries of the ranges for the $\frac{\Delta A}{A_0}$ values corresponding to these regimes are set by the critical values of the area asymmetry, $\left(\frac{\Delta A}{A_0}\right)^*$

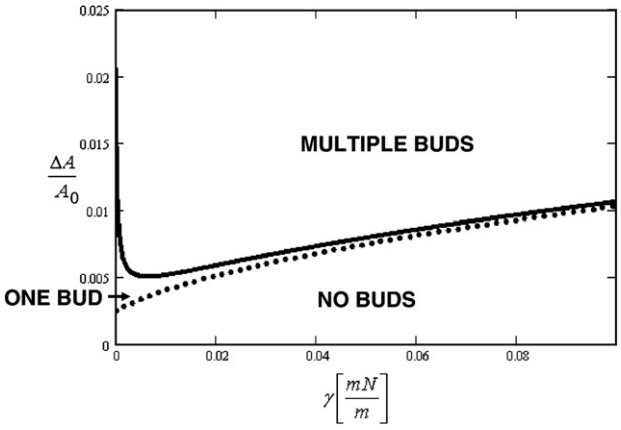

Fig. 6. Phase diagram in terms of the monolayer area asymmetry and membrane tension. Three regions separated by phase boundaries can be distinguished: a region of no vesicles forming; a single vesicle region; a multiple vesicle region. The lower and upper phase boundaries are determined by the equations Eqs. (A6) and (A7), respectively, using the parameter values $\kappa_B = 8\cdot10^{-20}$ J; $\Gamma = 100$ mN/m; $\delta = 2$ nm; $R = 500$ nm.

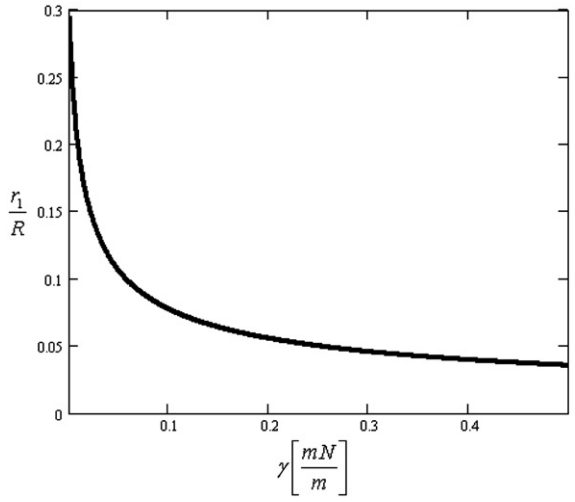

Fig. 7. Radius of the vesicle formed in the single vesicle regime as a function of the lateral tension. The parameter values are those of Fig. 6 and the values of $\Delta A/A_0$ correspond to the lower phase boundary of the phase diagram.

and $\left(\frac{\Delta A}{A_0}\right)^{**}$, which are determined by the membrane lateral tension γ and the elastic parameters of the membranes (see Appendix).

1. As long as the monolayer area asymmetry is small,

$$\frac{\Delta A}{A_0} < \left(\frac{\Delta A}{A_0}\right)^{*} \tag{2}$$

no vesicle is formed.

2. For the monolayer area asymmetry varying between the two critical values

$$\left(\frac{\Delta A}{A_0}\right)^{*} < \frac{\Delta A}{A_0} < \left(\frac{\Delta A}{A_0}\right)^{**}, \tag{3}$$

only one vesicle is formed. This will be referred to as a single vesicle regime.

3. Finally, for larger monolayer area asymmetry,

$$\frac{\Delta A}{A_0} > \left(\frac{\Delta A}{A_0}\right)^{**}, \tag{4}$$

many vesicles are generated, which will be called the multiple vesicle regime.

The three regimes can be illustrated by a phase diagram (Fig. 6), which represents the monolayer area asymmetry $\Delta A/A_0$ corresponding to each regime as a function of the lateral tension γ. The specific parameter values used for calculating the phase diagram in Fig. 6 are: the bilayer bending rigidity $\kappa_B = 8 \cdot 10^{-20}$ J, which is based on the characteristic value of the monolayer bending rigidity of $\kappa = 4 \cdot 10^{-20}$ J [72] and implying a zero monolayer Gaussian modulus, $\bar{\kappa} = 0$; the monolayer stretching modulus $\Gamma = 100$ mN/m (see [73]); the monolayer thickness $\delta = 2$ nm; and the vesicle radius $R = 500$ nm. The phase diagram (Fig. 6) demonstrates that the larger the tension γ, the larger monolayer area asymmetry $(\Delta A/A_0)^*$ is needed to generate a vesicle. This could explain the experimental results on modulation of endocytosis by osmotic pressure according to which formation of endocytic vesicles can be slowed down [74] or even stopped [75] by imposing a transmembrane pressure (and, hence the related membrane tension), and recovered by increase of the area asymmetry [75]. However, the dependence of the critical area asymmetry $(\Delta A/A_0)^*$ on the tension γ is relatively weak so that the required $(\Delta A/A_0)^*$ remains in a feasible range of less than one percent for relevant tensions up to 0.1 mN/m (Fig. 6). Remarkably, the range of $\Delta A/A_0$ corresponding to the single vesicle regime is infinitely large at vanishing tensions ($\gamma \to 0$), but becomes very narrow for tensions larger than $\sim 10^{-3}$ mN/m. Hence, the model predicts that in the absence of lateral tension the monolayer area asymmetry can only generate a single vesicle, while application of sufficient $\Delta A/A_0$ in the presence of a low lateral tension γ results practically always in the generation of multiple vesicles.

Within the single vesicle regime the vesicle radius r depends on the monolayer area asymmetry $\Delta A/A_0$, the dimension of the initial membrane, R, and the membrane tension γ (see Appendix). The vesicle radius decreases with growing γ (Fig. 7) but, importantly, remains comparable with the dimension of the donor membrane. Only for tensions considerably larger than the biologically relevant ones r reaches a value of a few percents of R.

In contrast, in the multiple vesicle regime the vesicle radius r does not depend on the value of the monolayer lipid asymmetry $\Delta A/A_0$ (with the only requirement that the latter has to be above a critical value, Fig. 6) and is determined only by the value of the lateral tension γ and the membrane bending rigidity κ_B according to

$$r = \sqrt{2\kappa_B/\gamma} \tag{5}$$

(see Appendix).

Plasma membranes of cells are, in most cases, subjected to lateral tension, which results from two major factors — the transmembrane osmotic pressure [70,71], and the forces produced by actin filaments polymerizing against the membrane. The tension generated by osmotic pressure varies in the range between 0.01 and 0.05 mN/m [76]. The actin induced tension should be somewhat larger. Taking into account that 1 μm of the leading edge of a moving cell is pushed by hundreds of actin filaments each of which generating a few pN force [77], one expects a membrane tension in the range of 0.1–1 mN/m. Our model predicts (Fig. 6) that if a tension of about 0.1 mN/m is applied to the bilayer, the monolayer area asymmetry results in generation of multiple small vesicles with a dimension of a few tens of nanometers, which is characteristic to intracellular vesicles. Importantly, in this case the vesicle radius (Eq. (5)) is independent of the dimension R of the donor membrane meaning that multiple small vesicles can form out of large membranes. Hence, such a vesiculation mechanism is biologically feasible and may contribute to the formation of the intracellular transport vesicles during the first step of endocytosis. This mechanism of vesicle formation can be complementary to the mechanisms of the endocytic vesicle formation based on the action of specialized proteins [49,50]. For example, flippase mediated lipid transport leading to the monolayer area asymmetry may cause an initial membrane bending which could be reinforced and promoted by curvature sensing proteins like the BAR domains. Eventually, the concerted action of such proteins and the monolayer area asymmetry should lead to robust membrane budding and vesiculation.

Taken together, our analysis predicts that the interplay between the area asymmetry and the membrane lateral tension can generate multiple small vesicles whose size is determined by the tension value. Importantly, for the biologically relevant tensions the forming vesicles are predicted to be as small as the endocytic vesicles, which implies that the monolayer lipid asymmetry can contribute to endocytosis and, possibly, to the formation of membrane carriers from intracellular compartments.

In a biological perspective, membrane curvature can be generated by multiple mechanisms driven by specialized proteins [49,50,78]. For example, tight packing of integral membrane proteins with bulky ectodomains or scaffolding by oligomerisation of peripheral proteins such as COPI and COPII complexes and clathrin-related complexes into curved structures could generate local curvature. Insertion of N-terminal α-helices of BAR domain containing proteins and epsin is another

possible mechanism of membrane bending [49,50]. All these various mechanisms and the mechanism suggested here are not mutually exclusive and may act in concert or sequentially at different stages of membrane bending and formation of intracellular vesicles. For example, flippase mediated lipid transport leading to the monolayer area asymmetry may cause an initial membrane bending which could be reinforced and promoted by curvature sensing proteins like the BAR domains. Eventually, the concerted action of such proteins and the monolayer area asymmetry should lead to robust membrane budding and vesiculation.

8. Conclusion

In this review, we have shown the importance of membrane deformations that can be triggered by the translocation of a few lipids from one monolayer to the other in a lipid bilayer. This flip-flop process, which, normally, is very slow can become rapid in the presence of specific proteins called flippases. Actually, the membrane curvature behaves like an amplification factor of membrane structural changes because of the low percentage of molecules that are involved and yet give rise to an almost macroscopic effect, namely the formation of lipid vesicles able to wrap up many other molecules and, eventually, to transport them within the cell or out of the cell. The selectivity of the lipid translocation by a flippase enables cells to use selective lipids as messenger molecules for very diverse objectives. Notably, other mechanisms based on lipid organization could lead to bending and, eventually, budding. Using pure lipid mixture GUVs a correlation between fluid phase domain composition and membrane curvature has been reported [79] in agreement with theoretical predictions [80]. Long-range domain ordering of various patterns of circular domains, curvature-dependent domain sorting, and membrane fission into separate vesicles at domain boundaries have been observed.

The point we have emphasized in this review and which is unusual in molecular biology is that the messenger molecules are not simply recognized by a receptor. Instead, a change of the whole membrane structure takes place, which is secondary to the formation of vesicles or the stimulation of an enzyme with a lipid co-enzyme. The lipid heterogeneity, which exists in cell membranes, opens the possibility to control different mechanisms within the same lipid bilayer. It is therefore a progress of the evolution.

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Appendix

Physical model of membrane vesiculation by the monolayer area asymmetry upon lateral tension

A complete analysis of membrane shapes generated by the monolayer area asymmetry upon lateral tension would require a thorough numerical treatment similar to that performed for vesicles with constant area and volume [81]. Here we limit ourselves by seeking for conditions of membrane vesiculation, and determine the vesicle number and dimensions. Therefore, as a state alternative to the initial flat configuration we consider the vesiculated state consisting of n identical spherical vesicles and the remaining flat bilayer (Fig. A1). The mid surfaces of the vesicular membranes have a radius r (total curvature $J=2/r$).

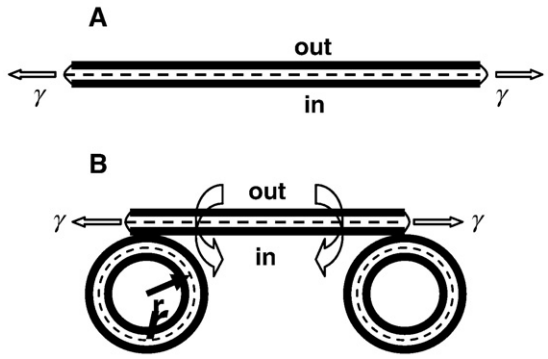

Fig. A1. Illustration of the model. A.) Initial state: flat bilayer subjected to lateral tension. B.) Vesiculated state: formed vesicles and the remaining flat bilayer. The bent arrows show the direction of lipid transfer by flippases.

We will determine the number n of the forming vesicles and their radius r by minimizing the total energy of the system, F_{tot}, which includes the energy related to the lateral tension, F_γ, in addition to the stretching–compression F_{STR} and the bending, F_{B}, energies of the membrane monolayers determined by the interplay between the monolayer area asymmetry and the membrane curvature.

Energy of monolayer stretching–compression. In the initial flat state (Fig. A1A), the membrane accumulates the monolayer stretching–compression energy related to the difference of their spontaneous areas.

In the vesiculated state with n vesicles of radius r and curvature $J=2/r$ (Fig. A1B) the system is characterized by the surface average of the total curvature

$$\langle J\rangle = \frac{n\cdot 4\pi\cdot r^2\cdot 2/r}{4\pi\cdot R^2} = \frac{2\cdot n\cdot r}{R^2}, \tag{A1}$$

which reduces the monolayer stretching–compression deformations. The monolayer stretching–compression energy of the vesiculated state related to that of the initial flat state, is given by

$$F_{\text{STR}} = 16\pi\cdot\Gamma\cdot\delta\cdot n\cdot r\cdot\left(\frac{n\cdot r\cdot\delta}{R^2} - \frac{\Delta A}{A_0}\right), \tag{A2}$$

where δ is the monolayer thickness.

Bending energy. Bending energy of one vesicle is the sum of the bending energies of its monolayers. In the small curvature approximation, $|J\cdot\delta|\ll 1$, and for zero monolayer spontaneous curvature, the bending energy of one vesicle equals $F_{\text{B}}^{\text{vesicle}} = 8\,\pi\cdot\kappa_{\text{B}}$, where $\kappa_{\text{B}} = 4\kappa + 2\kappa$ accounts for the bending, κ, and Gaussian curvature, κ, moduli of the two monolayers. The total bending energy in the vesiculated state as compared to that in the initial flat state is

$$F_{\text{B}} = n\cdot 8\pi\cdot\kappa_{\text{B}}. \tag{A3}$$

Energy of tension. The energy of tension corresponding to transition between the initial and vesiculated states is $F_\gamma = \gamma\cdot n\cdot A_{\text{v}}$, where A_{v} is the mid surface area of a vesicle. This expression means that the remaining flat bilayer plays a role of an effective lipid reservoir, which keeps the tension γ constant upon vesiculation. Generation of n vesicles is accompanied by pulling the area $n\cdot A_{\text{v}}$ out of this effective reservoir and, hence, requires the energy above, which can be expressed through the vesicle radius r by

$$F_\gamma = 4\pi\cdot\gamma\cdot n\cdot r^2. \tag{A4}$$

The total energy of transition between the flat (Fig. A1A) and vesiculated (Fig. A1B) states is

$$F_{\mathrm{tot}} = 16\pi \cdot \Gamma \cdot \delta \cdot n \cdot r \cdot \left(\frac{n \cdot r \cdot \delta}{R^2} - \frac{\Delta A}{A_0}\right) + 4\pi \cdot \gamma \cdot n \cdot r^2 + n \cdot 8\pi \cdot \kappa_{\mathrm{B}}. \tag{A5}$$

Minimization of the energy Eq. (A5) with respect to the vesicle radius, r, and the vesicle number, n, determines the values of the monolayer area asymmetry, $\Delta A/A_0$, necessary for vesiculation, and predicts the corresponding dimensions and numbers of the vesicles. There are different regimes of vesiculation determined by two critical values of the monolayer area asymmetry:

$$\left(\frac{\Delta A}{A_0}\right)^{*} = \sqrt{2\frac{\kappa_{\mathrm{B}}}{\Gamma\delta^2}\left(\frac{\delta^2}{R^2} + \frac{1}{4}\cdot\frac{\gamma}{\Gamma}\right)} \tag{A6}$$

and

$$\left(\frac{\Delta A}{A_0}\right)^{**} = \sqrt{8\frac{\kappa_{\mathrm{B}}}{\Gamma\delta^2}}\left(\frac{\delta^2}{R^2} + \frac{1}{4}\cdot\frac{\gamma}{\Gamma}\right). \tag{A7}$$

Discussion of the single vesicle and multiple vesicle regimes and the related phase diagram are presented in the main text.

Vesicle number and dimension. Within the single vesicle regime the vesicle radius r_1 depends on the monolayer area asymmetry $\Delta A/A_0$, and the dimension of the initial membrane, R, according to

$$r_1 = \frac{1}{2}\cdot\frac{\delta}{\left(\frac{\delta^2}{R^2} + \frac{1}{4}\cdot\frac{\gamma}{\Gamma}\right)}\cdot\frac{\Delta A}{A_0}. \tag{A8}$$

According to the dependence of r_1 on $\Delta A/A_0$, for R=500 nm and γ=0.001 mN/m, the vesicle radius, r_1, is just a few times smaller than that of the donor membrane, R. Dependence of r_1 on the lateral tension γ is illustrated by Fig. 7 of the main text. The vesicle radius decreases with growing γ and only for tensions considerably larger than the biologically relevant ones it reaches a value of a few percents of R.

Summarizing, in the one vesicle regime the vesicle dimension is comparable with the dimension of the donor membrane.

Within the multiple vesicle regime, the number n of forming vesicles depends on the system parameters according to

$$n = \frac{R^2}{\delta^2}\cdot\left(\sqrt{\frac{1}{8}\cdot\frac{\gamma\cdot\delta^2}{\kappa_{\mathrm{B}}}}\cdot\frac{\Delta A}{A_0} - \frac{1}{4}\cdot\frac{\gamma}{\Gamma}\right). \tag{A9}$$

The vesicle number grows linearly with the monolayer area asymmetry $\Delta A/A_0$. Dependence of the vesicle number on the tension has a non-monotonic character, the maximal number of vesicles corresponding to $\gamma = \frac{1}{2}\cdot\frac{\Gamma\cdot\delta^2}{\kappa_{\mathrm{B}}}\cdot\left(\frac{\Delta A}{A_0}\right)^2$.

The radius of one vesicle formed in the multiple vesicle regime is determined by

$$r = \sqrt{2\cdot\frac{\kappa_{\mathrm{B}}}{\gamma}}. \tag{A10}$$

In contrast to the single vesicle regime, in the multiple vesicle regime the vesicle radius is determined only by the value of the lateral tension γ and the membrane bending rigidity κ_{B}. At biologically feasible tensions around 0.1 mN/m the vesicle radius reaches the value of about 50 nm typical for endocytic vesicles.

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