Enhancement of endocytosis due to aminophospholipid transport across the plasma membrane of living cells

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Enhancement of endocytosis due to aminophospholipid transport across the plasma membrane of living cells. Am. J. Physiol. 276 (Cell Physiol. 45): C725–C733, 1999.—Formation of intracellular vesicles is initiated by membrane budding. Here we test the hypothesis that the plasma membrane surface area asymmetry could be a driving force for vesicle formation during endocytosis. The inner layer phospholipid number was therefore increased by adding exogenous aminophospholipids to living cells, which were then translocated from the outer to the inner layer of the membrane by the ubiquitous flippase. Addition of either phosphatidylserine or phosphatidylethanolamine led to an enhancement of endocytosis, showing that the observed acceleration does not depend on the lipid polar head group. Conversely, a closely related aminophospholipid that is not recognized by the flippase, lyso-α-phosphatidylserine, inhibited endocytosis, and similar results were obtained with a cholesterol derivative that also remains in the plasma membrane outer layer. Thus an increase of lipid concentration in the inner layer enhanced internalization, whereas an increase of the lipid concentration in the outer layer inhibited internalization. These experiments suggest that transient asymmetries in lipid concentration might contribute to the formation of endocytic vesicles.

cloathrin; membrane budding; flippase

Endocytosis of Plasma Membrane Components and Molecules from the Extracellular Environment is Important for the Function of Eukaryotic Cells. Endocytosis involves the formation of pits in the membrane that invaginate and form vesicles that bud from the plasma membrane. The best-characterized endocytic pathway involves clathrin-coated pits and vesicles. Receptors, such as the transferrin (Tf) receptor (TfR), concentrate in these pits and vesicles.

The budding force leading to membrane vesiculization in endocytosis is often thought to be generated by the polymerization of clathrin at the plasma membrane (31). However, several experiments indicate that there may not be a strict correlation between membrane budding and clathrin polymerization. Thus increasing the amount of polymerized clathrin at the plasma membrane did not increase the number of invaginated coated pits (23), and preventing the formation of clathrin vesicles did not inhibit bulk flow endocytosis of plasma membrane components (5, 32). These results therefore raise the question of what may be the force causing membrane budding and vesicle formation (18).

Different alternative mechanisms, relying on the physical elastic properties of the plasma membrane, have been proposed for the budding force. An increase in the net surface area of the plasma membrane, the local segregation of phospholipid domains, or a difference in the surface area between the inner and outer layers of the membrane could all theoretically induce vesicle formation (14, 22, 33). The three types of perturbation were in fact shown to induce vesicle formation in liposomes (7, 10, 15). However, the hypothesis of net surface area increase is only applicable for liposomes characterized by a well-defined internal volume and flat surface membrane, which is far from being the case for plasma membranes of living cells. On the other hand, the lipid domain hypothesis has the advantage of successfully predicting the formation of 100- to 400-nm vesicles (14, 17), but the existence of phospholipid microdomains in the plasma membranes of living cells still remains to be clearly demonstrated (25). Experiments performed to test the possibility that the phospholipid bilayer itself could generate the budding force in living cells have therefore focused on the surface area asymmetry hypothesis.

The latter mechanism is based on the fact that the curvature of a thick bilayer membrane is directly related to the surface area difference between its two monolayers (1, 35). Hence, a change in the surface area asymmetry leads to a membrane shape change, possibly resulting in vesicle formation (9). This mechanism was also supported by experiments on vesicle generation in red blood cells after a change in the plasma membrane phospholipid concentration asymmetry (27) and on vesiculization in cell-size liposomes in the absence of any protein involvement (10). Moreover, the dynamics of the early steps of bulk flow endocytosis of the phospholipid phosphatidylcholine (PC) were shown to be highly accelerated in living K562 cells in response to an increase in the phospholipid concentration in the inner layer of the membrane (8). This asymmetry was established after the addition of extraneous phosphatidylserine (PS) to the outer layer and its specific translocation to the inner layer by the endogenous flippase, a ubiquitous plasma membrane translocator of aminophospholipids (21, 34, 39).

In the present work, we characterized the dynamics of the early steps of endocytosis of plasma membrane proteins in response to the addition of different exogenous molecules: PS and phosphatidylethanolamine (PE), which are actively translocated to the inner layer of the membrane, or lyso-α-phosphatidylserine (l-PS) and poly(ethylene)50-glycol-cholesterol (PEG-50-Cho),
which remain in the outer layer. Bulk membrane proteins were biotinylated and coupled with FITC-streptavidin, and individual proteins were revealed with FITC-labeled antibodies. The time course of internalization of the fluorescently labeled proteins was monitored by following the fluorescence decrease of fluorescein, whose fluorescence is sensitive to pH and is thus quenched in the acidic environment of early endocytic vesicles. The internalization of the FITC-labeled proteins was also visualized by confocal microscopy. Bulk flow internalization was affected in a manner consistent with predictions; namely, molecules that increase the phospholipid number of the inner layer increase the rate of internalization, whereas those that increase the phospholipid number of the outer layer decrease their rate, in qualitative agreement with the model, suggesting that a plasma membrane phospholipid number asymmetry may produce a driving force for the budding in endocytosis.

**MATERIALS AND METHODS**

Cells and materials. K562, a human erythroleukemia cell line, was grown in suspension in RPMI 1640 with 10% decomplemented FCS and 2 mM L-glutamine. The short chain phosphatidylserine with six carbons on the second chain (C6-PS), a generous gift from Paulette Hervé (Institut de Biologie Physico-Chimique, Paris, France), was synthesized following a previously described protocol (11), and l-PS was purchased from Sigma (St. Louis, MO). Palmitoyl-C6-nitrobenzodiazole-PE (C6-PE) was purchased from Avanti Polar Lipids (Birmingham, AL). N-hydroxysuccinimidobiotin and streptavidin-FITC were purchased from Pierce (Rockford, IL). Human transferrin (Sigma) was loaded with iron (39). The cells were then cooled to 0°C to stop any endocytic activity before the procedure for labeling of the outer layer was initiated. The viability of the cells was determined by trypan blue exclusion after incubation of the cells with the above lipids under the same conditions as used for fluorescence measurements.

FITC-streptavidin-biotin labeling of surface membrane proteins. After incubation of the cells for 30 min with or without exogenous lipid and cooling of the mixture to 0°C, 4 mg/ml N-hydroxysuccinimidobiotin was added for 30 min on ice. The cells were then washed three times in 1 ml PBS at 0°C. The biotinylated cells were diluted in 500 µl PBS and incubated for another 30 min on ice with 7.5 µl of fluorescein-conjugated streptavidin at 1 mg/ml. The cells were washed three times in 1 ml cold PBS and then resuspended in a final volume of 350 µl PBS on ice.

Labeling of αY expressed on the surface of transfected K562 cells with a fluorescein-conjugated α-chain monoclonal antibody. Antibody (1.5 µg/ml) was added for 1 h on ice to the cells that had been incubated with C6-PS. After three washes in 1 ml cold PBS, the cells were resuspended in a final volume of 350 µl PBS on ice.

Endosomal distribution analyzed by confocal microscopy. Either wild-type K562 cells or K562 cells stably transfecte
medium consisting of 3.7% paraformaldehyde in 0.03 M sucrose. Fixation was allowed to proceed for 30 min on ice, and the reaction was quenched for 10 min with 50 mM NH₄Cl in PBS at room temperature. After two washes in PBS, the sample was resuspended in 25 mg/ml DABCO [1,4-diazabicyclo(2.2.2)octane; Sigma] and 100 mg/ml MOWIOL (Calbiochem, La Jolla, CA) (29). This solution was then mounted on microscope slides.

The samples were examined with a confocal microscope (Leica) attached to a diaplan microscope (Leitz) equipped with an argon-krypton double laser. Serial optical sections were recorded at 0.5-µm intervals with a ×63 lens. Photographs were taken on Kodak Ektachrome 100.

RESULTS

Early steps of internalization into acidic compartments monitored by the fluorescence quenching of plasma membrane proteins labeled with fluorescein. The fluorescence quenching of fluorescein due to transfer of the fluorochrome from a physiological pH of 7.4 to the acidic environment of early endocytic compartments (36) has previously been exploited to follow the internalization of the epidermal growth factor by endocytosis (2). This probe has an acidic dissociation constant (pKₐ) value (pKₐ = 6) that maximizes the sensitivity of endocytosis kinetics measurements based on the quenching of the fluorescent fluorochrome.

This strategy was used here to monitor the dynamics of the early steps of bulk flow endocytosis and receptor-mediated endocytosis. The validity of this approach was first determined by studying internalization of the TfR, which is endocytosed through the coated-pit pathway. In most cell types, this receptor is internalized with a half time of 2.5 min; internalization is followed by recycling through the cell with a complete exit time of 12.5 min (3, 6). We thus measured the fluorescence quenching kinetics of an FITC-labeled Tf. Figure 1 shows that the fluorescence varies as a function of time with two different profiles. First, the fluorescence is quenched, with maximal quenching observed at 2.7 min. The quenching is then followed by recovery of the fluorescence, which reaches a plateau after 12.5 min. Fluorescence quenching is due to Tf internalization into acidic compartments, and the recovery most likely corresponds to receptor recycling to the surface; these results are thus in good qualitative and quantitative agreement with the TfR recycling kinetics previously determined by other methods (3).

We next followed bulk flow endocytosis by measuring the fluorescence quenching of FITC-streptavidin-biotin-treated cells. To ascertain that the fluorescence quenching corresponds to bulk flow endocytosis of fluorescent proteins, we compared the fluorescence quenching of FITC-streptavidin-biotin-treated cells at 37 and 7°C. At 7°C, a temperature at which endocytosis is inhibited, there is essentially no fluorescence quenching of the labeled membrane proteins in the absence of endocytosis (Fig. 2A). In contrast, there is significant fluorescence quenching at 37°C, with a half time of 3 min. Thus, at 37°C, the fluorescein-labeled surface proteins are actively internalized by the cell.

Finally, to verify that the fluorescence quenching is in fact due to internalization into acidic compartments, we added monensin to the cells after surface protein internalization had proceeded for 10 min (Fig. 2B). Monensin, a monovalent ion-selective ionophore, exchanges sodium ions for protons and is known to incorporate into plasma and intracellular membranes immediately after incubation (24). Consequently, it equilibrates the pH between the extracellular medium and all internal compartments, resulting in a neutral pH throughout. Due to pH neutralization of the endosomes, the initial fluorescence intensity should therefore be recovered. When FITC-streptavidin-biotin-labeled cells were first incubated in the cuvette at 37°C and 1% monensin was added after 10 min, fluorescence quenching was observed from 0 to 10 min, followed by rapid recovery of the fluorescence after monensin addition (Fig. 2B). This result demonstrates that the acidic pH of the internal compartments is necessary for fluorescence quenching.

Incubation of fluorescein-labeled cells with C6-PS or C6-PE induces acceleration of bulk flow endocytosis. We wished to determine whether the creation of a phospholipid number asymmetry between the two leaflets of the plasma membrane may generate enough of a driving force to promote budding of endocytic vesicles (9). We therefore took advantage of the endogenous flippase activity of the cell, which translocates aminophospholipids from the outer layer to the inner layer of the plasma membrane (34, 39), to generate an increase of the phospholipid number asymmetry.

C6-PS was added to the outer layer of the plasma membrane. This aminophospholipid is recognized specifically by the flippase and is translocated into the inner layer within 30 min at 37°C in the K562 cell line (4). Under these conditions, translocation leads to a steady state in which typically 80–90% of the added PS is translocated to the inner layer of the plasma mem-
brane while 10–20% remains in the outer layer (4, 8). The amount of C6-PS added was previously calibrated in comparison with values of the line width broadening of spin-spin interactions in electron spin resonance spectroscopy experiments (8, 19): 15 nmol of C6-PS correspond to 1% of the outer layer phospholipid molecules of 10^8 cells. The cells were then cooled to 0°C and labeled with biotin followed by fluoresceinated streptavidin. After labeling, the kinetics of bulk flow endocytosis at 37°C were measured as a function of the percentage of C6-PS added to the cells.

Figure 3A shows the increase in endocytosis measured after adding C6-PS to the cells. The amount of labeled plasma membrane proteins internalized at 10 min was increased by a factor of from two (for 2% C6-PS added) to four (for 4% C6-PS added) compared with the amount internalized in the absence of C6-PS (0% C6-PS).

Figure 3B shows the initial slope of the bulk flow endocytosis time course as a function of the percentage of added PS. A mean linear dependence of the initial slope on the percentage of added PS was observed. The characteristic slope of this dependence was found to be 1.1 ± 0.4%/min·%PS⁻¹. This corresponds to an increase of a factor of about five in the bulk flow endocytosis kinetics for an addition of 4% C6-PS.

To verify whether the enhancement in the rate of fluorescence quenching after addition of C6-PS was due to internalization of the FITC moiety into acidic compartments, monensin was added after 10 min of measurement (at 37°C) of cells that had been preincubated with 2% PS. Addition of monensin increased the fluorescence to initial levels (not shown), implying that the...
exogenous PS had increased the rate of entry into acidic compartments.

Finally, fluorescence microscopy experiments were performed to directly observe the increase in endocytosis kinetics. The protocol for C6-PS incubation and plasma membrane labeling with fluorescein was followed as described above, and, after endocytosis was allowed to proceed at 37°C, the cells were cooled to 0°C, fixed with paraformaldehyde, and mounted on microscope slides for analysis by confocal microscopy. A concentration of 2.5% C6-PS was chosen, since under these conditions the internalization kinetics increase by a factor of three (Fig. 3B), and endocytosis was stopped after 6.5 min, at which time there is a significant amount of internalization (Fig. 3A).

The confocal micrographs in Fig. 4 show fluorescein internalization into endocytic compartments with and without previous incubation with 2.5% C6-PS. For cells that had not been incubated with C6-PS (Fig. 4, left), the fluorescence labeling is located mainly at the plasma membrane, with poor intracellular labeling. In contrast, the cells incubated with C6-PS effectively showed significant labeling of intracellular compartments, in addition to the plasma membrane (see Fig. 4, right). The effects of C6-PS observed by confocal microscopy thus qualitatively confirmed the fluorescence quenching experiment observations.

To rule out the possibility that the effects of C6-PS may be due to biochemical properties of the anionic PS head group, these experiments were repeated with another aminophospholipid that is translocated to the inner layer of the membrane, the short chain C6-PE, which has a neutral charge at neutral pH (41). After incubation of the cells with 2% C6-PE under the same experimental conditions as for C6-PS, there is an increase in the rate of internalization (Fig. 5A), which is comparable to the effect of C6-PS.

Translocation of PS into the inner layer of the plasma membrane is required for acceleration of bulk flow endocytosis. Because incubation of cells with C6-PS increases bulk flow endocytosis, we determined whether it was necessary for the short chain PS to be translo-

![Fig. 4](image1.png) Enhance
ment of bulk protein endocytosis following addition of C6-PS, as observed by confocal microscopy. Cells were incubated at 37°C without (left) and with (right) 2.5% C6-PS, and cell surface proteins were labeled with fluorescein on ice, as described in MATERIALS AND METHODS. Cells were then transferred into PBS at a final temperature of 37°C and fixed after 6.5 min of incubation as described in MATERIALS AND METHODS. A representative medial optical section is shown. Bar, 10 µm.

![Fig. 5](image2.png) Enhancement of bulk protein endocytosis after addition of short chain phosphatidylethanolamine (C6-PE) and inhibition after addition of lyso-α-phosphatidylserine (l-PS) or poly(ethylene)glycol-cholesterol (PEG-50-Cho). A: cells were first incubated at 37°C with 2% C6-PE, and cell surface proteins were labeled with fluorescein on ice, as described in MATERIALS AND METHODS. Cells were then transferred into a spectrofluorometer cuvette at a final temperature of 37°C, and endocytosis was monitored by following fluorescence quenching as a function of time. B and C: cells were first incubated at 37°C with l-PS (B) or PEG-50-Cho (C), and cell surface proteins were labeled with fluorescein on ice, as described in MATERIALS AND METHODS. Cells were then transferred into the spectrofluorometer cuvette at a final temperature of 37°C, and endocytosis was monitored by following fluorescence quenching as a function of time (n = 3).
located from the outer to the inner layer of the plasma membrane for this acceleration to be observed. Hence, we used the single-chain I-PS instead of the short-chain C6-PS. The I-PS is not translocated across the plasma membrane by the flippase because part of the chain in the sn-2 position of the glycerol, which is recognized specifically by the flippase, is missing in the I-PS (42). The head group of I-PS is thus analogous to that of C6-PS, except that the lipid cannot be translocated to the inner layer of the plasma membrane.

The cells were incubated with 3% I-PS, and endocytosis was measured by spectrofluorometry. At this concentration of I-PS, which also had no effect on cell viability as determined by trypan blue exclusion (not shown), there was no enhancement in the rate of internalization (Fig. 5B). On the contrary, the basal rate of fluorescence quenching, corresponding to spontaneous internalization, was inhibited by the addition of I-PS. Thus increasing the number of lipids on the outer layer of the membrane decreased the rate of internalization. Increasing the outer layer surface area with PEG-50-Cho inhibits bulk flow internalization. Given the inhibitory effect of I-PS, we evaluated whether increasing the surface area of the outer layer with a structurally unrelated compound would also inhibit the rate of internalization. Hence, cells were preincubated with 2.5 μM PEG-50-Cho, since it was recently shown that, at this concentration, this cholesterol derivative inserts spontaneously into the outer layer of the membrane, and inhibits fluid phase endocytosis by HT-1080 cells (12). At the same concentration, there is significant inhibition of bulk flow internalization (Fig. 5C), further suggesting that an increase in the surface area of the outer layer decreases the rate of internalization.

C6-PS also increases the internalization rate of a receptor containing a clathrin-coated-pit localization signal. We have shown that bulk flow endocytosis is increased by C6-PS. To determine whether the PS may have an effect on clathrin-dependent endocytosis, we used a stably transfected cell line that expresses αY, an IL-2 receptor α-chain that has been modified to contain the clathrin-coated-pit localization signal of the TfR (37). We did not use Tf for these experiments, due to its fast recycling time (see Fig. 1). An FITC-labeled anti-α-chain antibody was used to follow specifically the internalization of αY, in cells that had the added advantage of expressing low levels of αY (37). Figure 6 shows that the internalization of αY is increased significantly due to preincubation with 4% C6-PS. It should be noted, nonetheless, that the internalization of αY was enhanced only by concentrations of exogenous PS ≳ 3%, suggesting that the responsiveness to a membrane lipid asymmetry is different for bulk flow internalization and internalization of a receptor containing a clathrin-dependent endocytosis motif.

Confocal microscopy was also used to observe αY endocytosis in this cell line, using the FITC-labeled anti-α-chain antibody. Endocytosis was allowed to proceed for 2.5 min before the cells were quickly cooled and fixed. This time was chosen because most of the difference in fluorescence quenching between the C6-PS-stimulated and unstimulated cell samples takes place by 2.5 min (Fig. 6). As seen in Fig. 7, in the absence of C6-PS, there is mostly plasma membrane labeling with the anti-α-chain antibody, with some fluorescence internalization into endocytic vesicles near the plasma membrane. In contrast, there is a significant increase in intracellular labeling in the cells that had been preincubated with 4% C6-PS (Fig. 7, right), qualitatively confirming the result of the fluorescence quenching experiments shown in Fig. 6.

**DISCUSSION**

In the present work, we experimentally test the contribution that an asymmetry in the phospholipid concentration of the plasma membrane bilayer may have on early steps of endocytosis, on the assumption that the asymmetry may provide a sufficient physical

**Fig. 6.** Enhancement of clathrin-dependent endocytosis due to C6-PS, as observed by spectrofluorometry. Cells expressing αY, an interleukin-2 receptor α-chain containing the clathrin-coated-pit localization signal of TfR, were incubated at 37°C with 4% C6-PS and labeled with a fluorescein-labeled antibody on ice, as described in MATERIALS AND METHODS. They were then transferred into PBS in a spectrofluorometer cuvette at a final temperature of 37°C, and endocytosis was monitored by following fluorescence quenching as a function of time (n = 3).

**Fig. 7.** Enhancement of clathrin-dependent endocytosis due to C6-PS, as observed by confocal microscopy. Cells expressing αY were incubated at 37°C without (left) and with (right) 4% C6-PS, and cell surface proteins were labeled with fluorescein-labeled antibody on ice, as described in MATERIALS AND METHODS. Cells were then transferred into a PBS solution at a final temperature of 37°C and fixed after 2.5 min of incubation as described in MATERIALS AND METHODS. A representative medial optical section is shown. Bar, 10 μm.
driving force for membrane budding and hence vesicle formation.

A change in the curvature of membranes induces a surface area difference between the two leaflets of the bilayer, and, conversely, a difference in the surface area between the two leaflets induces a curvature of the bilayer, potentially leading to vesicle formation (1, 35). These predictions have been confirmed for red blood cells (27), which are not capable of endocytosis, and cell-size liposomes devoid of proteins (10), in which vesicles were observed at steady state after the creation of a surface area asymmetry. In addition, the kinetics of internalization of spin-labeled PC in K562 cells increased after the cells were incubated with exogenous PS, which was translocated to the inner layer of the plasma membrane by the endogenous flippase (8).

Here we show that the bulk flow endocytosis kinetics of membrane proteins are also accelerated after the addition and translocation of exogenous PS. The kinetics increased nearly threefold after the addition of 2% PS, and they increased linearly as a function of the amount of PS added, the initial slopes increasing about twofold with each percent of PS added.

To exclude the possibility that this acceleration may be due to increased acidification of the endosomal compartment due to phospholipid addition and translocation, we confirmed directly by confocal microscopy the enhancement of plasma membrane internalization.

The acceleration in bulk protein endocytosis may be compared with the value previously obtained for bulk lipid internalization in response to the same perturbation in phospholipid number transmembrane asymmetry. The kinetics of lipid internalization also increased linearly as a function of the amount of PS, increasing threefold after the addition of 2% PS (8). The good quantitative agreement between the values obtained for proteins and lipids with two different techniques thus confirms that membrane endocytosis can effectively be accelerated in response to addition and translocation of aminophospholipids. These results were confirmed with PE, an aminophospholipid having a different head group than PS.

To verify that the translocation of the PS onto the inner layer was necessary to generate the endocytosis enhancement, we performed experiments using I-PS instead of PS. I-PS, although it shares the same lipid head group moiety with C6-PS, has a short second acyl chain that precludes its recognition by the flippase. I-PS is thus not translocated into the inner layer of the membrane, and we found that it did not accelerate endocytosis showing that PS translocation to the inner layer is necessary to increase the endocytosis rate. On the contrary, we found that I-PS decreased the endocytosis rate, which is in agreement with the model of phospholipid number asymmetry as a driving force for budding, since increasing the phospholipid number in the outer layer should decrease the rate at which vesicles entering the cytosol are generated. Furthermore, the inhibitory effect of molecules staying in the outer layer did not depend on the molecular nature of the compound, since it was reproduced using a cholesterol derivative of polyethylene glycol, PEG-50-Cho, which remains in the plasma membrane outer layer.

We also considered the possibility that the internalization enhancement may correspond to a pathway that is independent of clathrin coat protein polymerization. We thus measured the internalization of αv, whose cytosolic tail contains a clathrin-dependent internalization signal, and found that this internalization was also accelerated by exogenous PS. This result is consistent with reports from other laboratories that clathrin polymerization by itself is not sufficient to induce budding of phospholipid bilayers (5) and that enhancement of clathrin polymerization does not increase the number of invaginated coated pits present near the plasma membrane (23). Furthermore, it suggests that the dynamic properties of the phospholipid bilayer may affect bending and budding of the clathrin lattice. However, we cannot exclude the possibility that the exogenous PS may be accelerating only a minor clathrin-independent component of αv internalization. Our data on fluorescence quenching of the fluorescent ligand of αv (anti-αv antibody) do not allow us to determine what fraction of the receptor is being internalized in the presence of exogenous PS.

It has been proposed that polymerization of coat protein might be required to concentrate specific protein ligands rather than to induce budding of the membrane bilayer (5, 23). The coat proteins may also be involved in later stages of membrane traffic, such as in preventing newly generated vesicles from immediately fusing again with the plasma membrane (38). Nonetheless, it may be premature to exclude a role for clathrin polymerization in the budding process, since the size of the coats often coincides with the size of clathrin-coated vesicles observed in electron micrographs (40, 43). It is thus conceivable that both coat polymerization and phospholipid bilayer dynamics may cooperate to give rise to membrane buds, and this conclusion is supported by the observation that higher concentrations of exogenous PS are required to enhance clathrin-dependent internalization than bulk flow internalization.

Local pH asymmetries could also contribute to the formation of intracellular vesicles. Given that these membranes contain the acidic phospholipid phosphatidic acid (13, 16), which is translocated from the acidic luminal monolayer to the basic cytosolic monolayer (30), the pH asymmetry could initiate the formation of cytosolic vesicles through its effect on the surface area asymmetry. This hypothesis is supported by the observation that liposomes containing acidic phospholipids give rise to small vesicles spontaneously in response to the creation of a transmembrane pH gradient (10). Moreover, the membranes in this liposome system also displayed continuous shape transitions ranging from oval shapes to elongated shapes with long tubular structures. Because the concentration of phosphatidic acid increases during endosomal maturation (13, 16), the surface area asymmetry could also increase during maturation, which could partially contribute to the development of endosomes ranging from quasispheres to elongated tubules.
There is apparently no pH asymmetry between the cytosolic and external sides of the plasma membrane. However, the ubiquitous flipase, which translocates aminophospholipids from the outer to the inner layer of the membrane, could play an equivalent role in vesicle formation. Our work effectively shows that, after addition of exogenous PS or PE to living cells and its specific translocation to the inner layer, there is a significant increase in the kinetics of both bulk flow and clathrin-dependent internalization of plasma membrane proteins. Although we have described experiments taking advantage of the endogenous flipase, it should be noted that many other processes within living cells could give rise to transmembrane asymmetries. Hence, the interaction between the external layer and phospholipases could stimulate vesicle formation by decreasing the area of the outer layer (44), and interactions between lipids and proteins such as clathrin components of coated pits or proteins of the cytoskeleton could increase the surface area of the inner layer, thus facilitating vesicle formation (20).

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