Increased Vesicle Endocytosis Due to an Increase in the Plasma Membrane Phosphatidylserine Concentration

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ABSTRACT Endocytosis vesiculation consists of local membrane invaginations, continuously generated on the plasma membrane surface of living cells. This vesiculation process was found to be activated in vivo by the generation of a transmembrane surface area asymmetry in the plasma membrane bilayer, after enhancement of transbilayer phospholipid translocation. The observed enhancement was shown to be in good quantitative agreement with a theoretical model of elastic equilibrium describing stabilization of 100-nm vesicles in response to phospholipid redistribution. Very rapid dynamic vesiculation and direct re-fusion of the vesicles, both dependent on the phospholipid translocation activity, were found on a time scale of seconds. Both vesiculation and re-fusion were shown to result in a steady-state population of internal vesicles at long time points. The plasma membrane appears to be a dynamic structure, oscillating between two distinct curvature states, the 10 μm^{-1} "vesicle" and the 0.1 μm^{-1} "plasma membrane" curvature states. This dynamic behavior is discussed in terms of an elastic control of the membranes curvature state by the phospholipid translocation activity.

INTRODUCTION

The membranes of living cells have remarkable elastic physical properties (Helfrich and Servuss, 1984; Lipowsky and Leibler, 1986; Mutz and Helfrich, 1989), which are believed to play a role in physiological processes. Moreover, these membranes move across the internal cell volume from one cellular compartment to another, by a succession of rapid vesiculation and fusion events (Steinman et al., 1983). These two basic membrane events constitutive of endocytosis (membrane internalization) and exocytosis (membrane secretion) still remain to be fully understood for living cells (Mellman, 1994). Concerning the membrane vesiculation during the initial steps of endocytosis, a quantitative model has been proposed based on an elastic curvature response of the membrane to translocation of phospholipids from the outer layer to the inner layer (Farge, 1994). The translocation depends on a phospholipid pumping activity previously described in biological membranes (Seigneuret and Devaux, 1984; Auland et al., 1994), which maintains membrane asymmetry but whose physiological function is unknown (for a review see Devaux, 1991). The relation between vesiculation and phospholipid translocation was supported by experiments that showed spontaneous budding and vesiculation in giant liposomes after a transmembrane phospholipid redistribution was induced (Farge and Devaux, 1992).

In this paper we report in vivo experiments supporting this model in living cells. We use the erythroleukemia cell line K562, wherein the translocation rate is proportional to the outer layer concentration of phosphatidylserine (PS), which is a specific substrate for the phospholipid pumping activity (Cribier et al., 1993). The phospholipid translocation is enhanced by the increase of the outer layer PS concentration, after addition of PS in the plasma membrane. As a result, the endocytic internalization of plasma membranes was observed to increase on a 10-min time scale. Moreover, on a seconds time scale, extremely rapid vesiculation and fusion fluctuations were measured, suggesting that the whole plasma membrane is a highly dynamic structure. These dynamics favored vesiculation asymmetrically. The amplitude of this asymmetry depended on the translocation activity and regulated endocytosis on a 10-min time scale.

THEORETICAL PREDICTIONS FOR ELASTIC VESICULATION

Considering a closed plasma membrane as two coupled elastic monolayers (Sheetz and Singer, 1974; Svetina and Zeks, 1989), the translocation activity is defined as a phospholipid redistribution of surface area δS_0 from one leaflet to the other. The resulting transmembrane surface area asymmetry induces a change in the elastic curvature state of the bilayer (Svetina and Zeks, 1989; Miao et al., 1991; Seifert et al., 1991). Such a shape change has in fact been shown to occur in nonbiological vesicles having typical radii $R_m \approx 10$ μm, which leads to the budding of vesicles, and is sometimes followed by their spontaneous fission (Farge and Devaux, 1992). In these experiments, only 0.1% of the membrane surface area needs to be translocated to trigger formation of a 5-μm-radius vesicle.

Theoretically, the triggering of one small vesicle of radius R_v should require the generation of a transmembrane surface area asymmetry ΔS_v, which is characteristic of a vesicle idealized as a sphere of surface area S_v, membrane

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thickness h, and curvature $1/R_v$. Simple geometry gives $\Delta S_v/S_v = 2h/R_v$. Consequently, a translocation of surface area giving rise to one vesicle should satisfy $\delta S_0/S_v = h/R_v$. This assumption is supported by theoretical considerations predicting the mechanical stabilization of vesicles when the radius R_v is small compared to the mean radius R_m of the membrane. In this case, the vesiculation follows simple geometrical constraints related to the surface area asymmetry characterizing a curved membrane, in response to the phospholipid translocation (Farge, 1994). However, in vivo the surface area asymmetry and the surface area endocytosed are not static parameters but rather dynamic ones, because the phospholipids are dynamically translocated by the pumping activity (Cribier et al., 1993) and vesicles are dynamically internalized by endocytosis. As a consequence, the translocation rate $\tau = d(\delta S_0)/dt$ should be related to the rate of vesiculation $\epsilon = dS_v/dt$ following the geometrical constraint condition, $d(\delta S_0)/dt = h/R_v dS_v/dt$. Thus the factor h/R_v provides a theoretical correlation for two rates: the endocytic rate and the translocation rate. In living cells, vesicles have a mean radius of 100 nm and a thickness of 5 nm (Alberts et al., 1994), so that $h/R_v \approx 5 \cdot 10^{-2}$. Directly applied to endocytosis in vivo, the geometrical model of vesiculation thus predicts a linear correlation between the rate of surface area translocation τ and the rate of endocytosis ϵ, $\tau/\epsilon \approx 5 \cdot 10^{-2}$.

MATERIALS AND METHODS

K562 cells

K562 cells were grown in RPMI 1640 culture medium, completed with 2 mM glutamine and 10% fetal calf serum. Cells were washed three times by low-speed centrifugation, and resuspended in fresh medium immediately before experiments.

Spin labels and ESR spectroscopy

The ESR experiments were performed on a Varian E109 spectrometer. The spin-labeled phospholipid analogs of phosphatidylcholine were synthesized following the method of Fellmann et al. (1994). The short-chain spin-labeled lipids solubilized in a chlorophorm/methanol solution (1:1, v/v) were dried in a glass tube under argon flow and then directly resuspended in complete fresh RPMI solution. The suspension was added to the cell solution to obtain a 1-ml solution at a final hematocrit of 50. Being surfactants, all of the short-chain lipids are transferred into the outer layer of the plasma membrane at the membrane phospholipid concentrations used here (Cribier et al., 1993; Israelachvili, 1991). The percentage of spin-labeled molecules added to the outer layer of the plasma membrane was evaluated by measuring the linewidth broadening due to the spin-spin interaction for concentrations that were typically $\gtrsim 1\%$ (Marsh and Smith, 1973).

Endocytosis kinetics measurements

Membrane internalization in K562 cells was monitored after a previously described protocol (Cribier et al., 1993), by adding 5% of a short-chain nitroxide spin-labeled phosphatidylcholine (PC) analog to the outer layer of the plasma membrane. As already shown in this protocol, spin-labeled PC does not cross the plasma membrane on the time scales investigated by us, and it can enter cells only if the membrane is internalized. However, the trigger of lipidic pores would require the addition of 30% short-chain lipids (Zhelev and Needham, 1995). The kinetics of membrane internalization is thus measured by how much of the spin-labeled probe is left on the outside membrane after different incubation times. This quantity is measured by bovine serum albumin (BSA) extraction of the outer layer probe fraction that had not been internalized by endocytic vesiculation. Briefly, after addition of the probes to the outer layer of the plasma membrane at time 0, 50-μl aliquots were taken from the cell solution at regular intervals of 2 min for the long time tail experiments, and of 10 s for the short time scale experiments. They were added to 120 μl of a 3% (w/v) BSA solution in fresh RPMI medium maintained at 4°C (Cribier et al., 1993). After 45 s at 4°C, any probe that had bound to the BSA was separated from the cells by centrifugation for 30 s at 7600 $\times$ g. The fraction of uninternalized probe was thus obtained at any incubation time by measuring the ESR spectrum intensity of the BSA/spin-label complex in solution. The kinetics were measured by adding the probes 45 min after the addition of the translocation substrate C6-PS to the cells (see below).

Correlation between PS concentration and translocation rate

A short-chain lipid, C6-PS (six carbons on the short chain), was used to increase the plasma membrane concentration of PS. C6-PS was dried and resuspended in medium solution as described above for the other short-chain lipids. This lipid has the advantage of incorporating spontaneously into the outer membrane layer after the addition of cells, and of having the right head-group composition to be translated by the endogenous pumping activity of the cell. Its incorporation and translocation were followed with spin-labeled short-chain PS analogs, using the BSA extraction method. In the case of K562 cells, PS was previously reported to be specifically translocated from the outer layer to the inner layer with a relative redistribution rate of 20% $\cdot$ min^{-1} of the outer layer PS at 37°C (Cribier et al., 1993). After 45 min of translocation, we checked that the steady-state regime was reached with typically 20% of the added PS remaining on the outer layer. In addition, we checked that the relative redistribution rate remained constant as a function of outer layer PS concentration increases that corresponded to $\leq 2\%$ of the plasma membrane surface area. The relative translocation rate decreased when the PS addition represented more than 2%, suggesting that the phospholipid pumping activity does not saturate below this critical PS concentration. The existence of a saturated translocation regime (70% of the added PS remained on the outer layer after 45 min, for PS addition of 10% of the plasma membrane area) ensures that the addition of PS does not favor the triggering of pores in the membrane, which would have on the contrary accelerated the translocation without any saturation effect. The critical value of 2% is in fact biologically relevant, because the natural PS concentration in natural plasma membranes is on the order of a few percent (Alberts et al., 1994). The experiments to enhance the net translocation rate of phospholipids by increasing the outer-layer PS concentration were therefore performed under nonsaturing translocation conditions, after 45 min of incubation of the cells incorporated with C6-PS. In this case, the net translocation rate is proportional to the outer-layer concentration by a factor of 0.2 min^{-1} (Cribier et al., 1993).

RESULTS

Experiments were performed at 37°C on the human erythroleukemia cell line K562. The specific substrate of the activity of phopholipid redistribution PS was used to accelerate the translocation rate. Because the relative translocation rate is 20% $\cdot$ min^{-1} of the PS present on the outer layer in these cells (Cribier et al., 1993), the net transmembrane translocation rate was enhanced by increasing the outer-layer PS plasma membrane concentration, which was done by adding PS directly to the cell outer layer. After a 45-min

incubation, the steady-state regime was reached, with 20% of the added PS remaining on the outer layer (see Materials and Methods). The outer-layer PS concentration therefore increased as a function of the PS added to the plasma membrane. Hence, all experiments were performed at steady state, after addition of PS from $\Delta C_{PS} = 0\%$ to $\Delta C_{PS} = 2\%$ of the plasma membrane surface area. The endocytic rate ϵ was measured at steady state as a function of the percentage of surface area of PS added to the plasma membrane ΔC_{PS}, allowing one to deduce the endocytic rate increase $\Delta\epsilon$ as a function of the net translocation rate increase $\Delta\tau$. The latter was calculated from the plasma membrane PS concentration increase ΔC_{PS} following the relation $\Delta\tau = 20\% \cdot 20\% \cdot \text{min}^{-1} \cdot \Delta C_{PS}$.

Membrane entry kinetics were first investigated on a 10-min time scale, which is generally used to study endocytosis (Subtil et al., 1995). Fig. 1 *A* shows an acceleration of membrane entry in response to an increase in the plasma membrane PS concentration ΔC_{PS}. The mean endocytic rate ϵ of the first 10 min was measured for three different experiments, at different PS concentration increases ΔC_{PS}. From these experiments emerge an enhancement of the membrane entry rate $\Delta\epsilon$ linearly correlated with ΔC_{PS} (Fig. 1 *B*). Deduced as a function of$\Delta\tau$, this simple linear relation is found to give $\Delta\tau/\Delta\epsilon = 2 \cdot 10^{-2} \pm 5 \cdot 10^{-3}$.

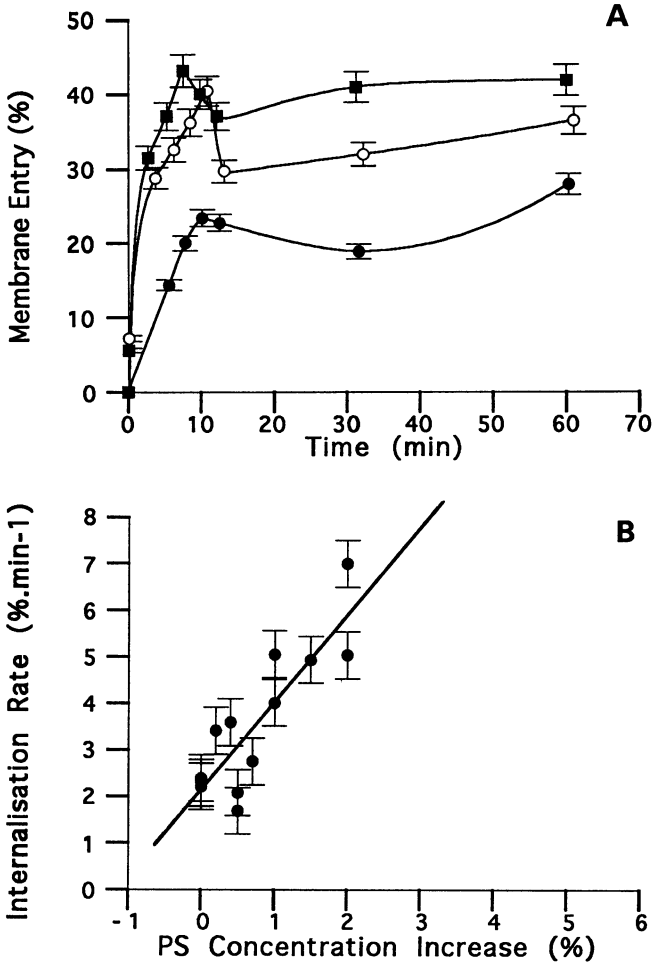

FIGURE 1 (*A*) Kinetics of endocytic membrane internalization of K562 cells as a function of the plasma membrane PS concentration increase ΔC_{PS}: ●, 0% PS; ○, 1% PS; ■, 2% PS. (*B*) The endocytic rate during the first 10 min as a function of the plasma membrane PS concentration increase ΔC_{PS}. Three distinct experiments are included. The scales depend on the percentage of the total plasma surface area. The correlation is linear, with a slope of $\Delta\epsilon/\Delta C_{PS} = 2 \pm 0.5\ \text{min}^{-1}$. From this slope is deduced a linear correlation between the membrane entry rate ϵ and the translocation rate τ of $\Delta\tau/\Delta\epsilon = 2 \cdot 10^{-2} \pm 5 \cdot 10^{-3}$ (see Results).

The very early steps of membrane entry were then analyzed on a shorter 10-s time scale. First, experiments were performed at a PS concentration normally found in natural membranes. Dynamic entry and exit of membrane oscillations displayed a large amplitude, typically of 10%, of the plasma membrane surface area (Fig. 2). As membrane entry is related to vesiculation, the membrane exit should be interpreted in terms of fusion of the vesicles with the plasma membrane. Given this interpretation, vesiculation (internalization) is therefore followed by refusion at this time scale. Furthermore, Fig. 2 exhibits a balance between membrane entry and membrane exit that is asymmetric. This balance should result in a net entry at long time intervals, which is observed in the internalization experiments of Fig. 1 *A* at a natural PS concentration.

These oscillations were also measured as a function of the plasma membrane PS concentration increase ΔC_{PS}, following the protocol for the long time period experiments. The amplitude oscillations exhibited an excitation in response to the increase in PS concentration, with both rapid entry and rapid exit being enhanced (see Fig. 3 *A*). A systematic analysis of the membrane entry rate and of the membrane exit rate was therefore performed for two different experiments, at different ΔC_{PS}. An increase in both the membrane entry ϵ_{ent} rate and the membrane exit ϵ_{exit} rate was in fact found, and both rates were linearly proportional to ΔC_{PS} (see Fig. 3 *B*). Deduced as a function of $\Delta\tau$, these gave $\Delta\tau/\Delta\epsilon_{ent} = 6.5 \cdot 10^{-3} \pm 10^{-4}$ and $\Delta\tau/\Delta\epsilon_{exit} = -1.2 \cdot 10^{-2}$

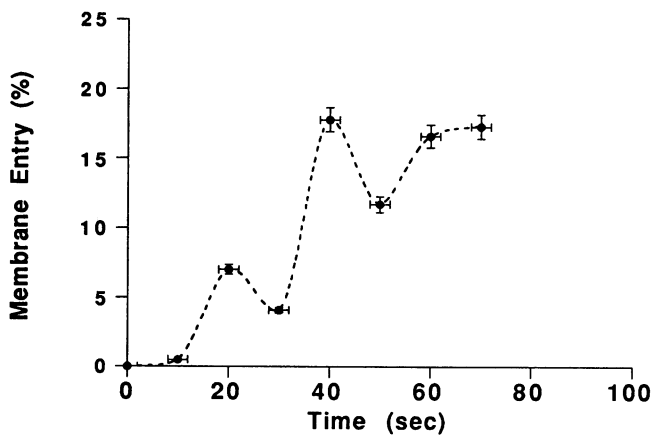

FIGURE 2 Internalization-exit membrane oscillations on the 10 s time scale at a natural plasma membrane concentration of PS. In this experiment, oscillation amplitudes correspond typically to 10% of the plasma membrane surface area.

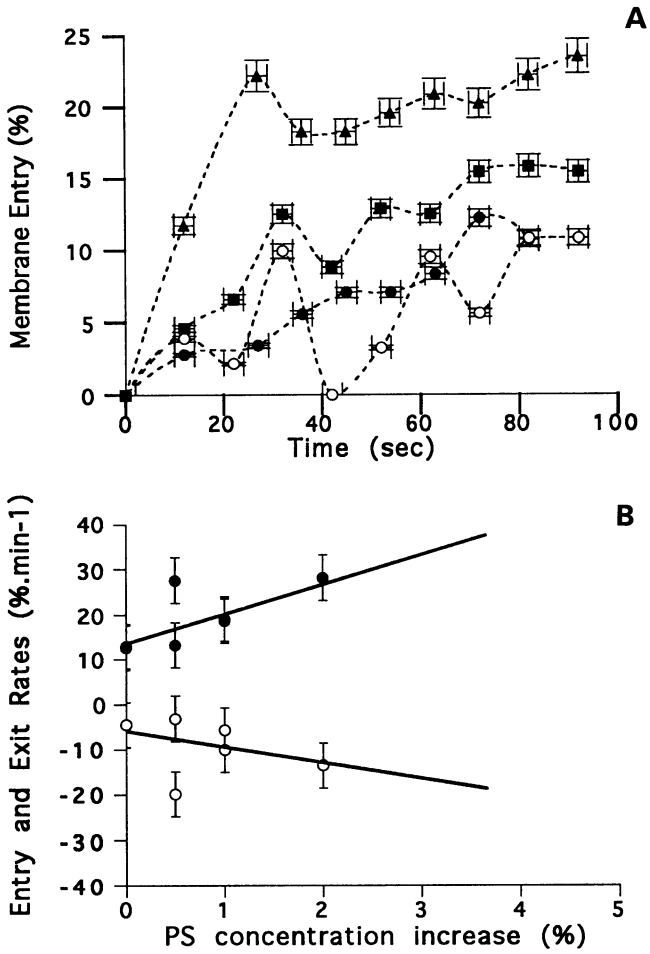

FIGURE 3 (*A*) Internalization-exit oscillations of K562 cells as a function of the plasma membrane PS concentration increase ΔC_{PS}: ●, 0% PS; ○, 0.5% PS; ■, 1% PS; ▲, 2% PS. (*B*) Internalization (●) and exit (○) rates on the 10 s time scale as a function of the plasma membrane PS concentration increase ΔC_{PS}. Two distinct experiments are included. The correlations are linear, with slopes of $\Delta\epsilon_{ent}/\Delta C_{PS} = 6.2 \pm 0.1$ min^{-1} and $\Delta\epsilon_{exit}/\Delta C_{PS} = -3.3 \pm 0.3$ min^{-1}, respectively. From these slopes are deduced a linear correlation between the membrane entry and exit rates and the translocation rate τ of $\Delta\tau/\Delta\epsilon_{ent} = 6.5 \cdot 10^{-3} \pm 10^{-4}$ and $\Delta\tau/\Delta\epsilon_{exit} = -1.2 \cdot 10^{-2} \pm 10^{-3}$, respectively (see results).

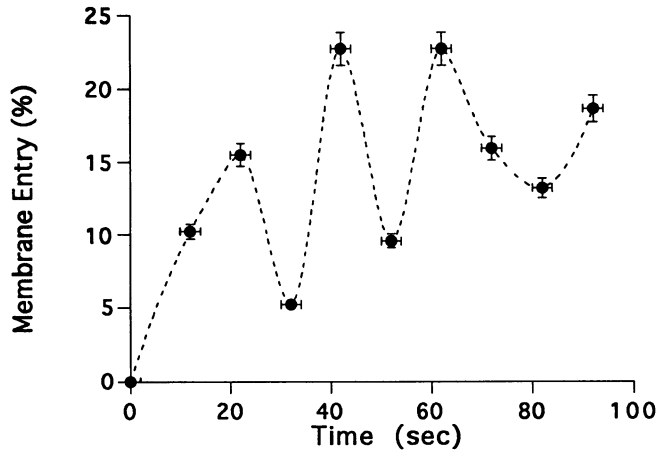

FIGURE 4 Internalization-exit oscillations of K562 cells for a plasma membrane PS concentration increase of $\Delta C_{PS} = 5\%$. Large entry-exit oscillation amplitudes of 20% of the total plasma membrane surface area can be triggered by displacing the translocation activity far from its steady-state regime.

$\pm 10^{-3}$. Interpreted in terms of vesiculation followed by rapid fusion in response to the translocation rate acceleration, the vesiculation thus increases twice as much as the fusion. Because of this asymmetry, one predicts a net increase of the entry rate at long time periods, correlated with the translocation rate activation by $\Delta\tau/\Delta\epsilon = 1.5 \cdot 10^{-2} \pm 10^{-3}$. This value is in good agreement with the value of $\Delta\tau/\Delta\epsilon = 2 \cdot 10^{-2} \pm 5 \cdot 10^{-3}$ found experimentally for the vesiculation rate enhancement at long time intervals.

DISCUSSION

Vesiculation regime at long time tail

The possibility that budding may arise simply from the elastic properties of the phospholipid bilayer has been extensively studied in the past few years by using nonbiological membranes. Three different models, supported by experiments on liposomes, were proposed. According to the first model, shape changes and budding are induced by changing the surface area-to-volume ratio of the closed membrane, which was experimentally improved on giant liposomes in response to a change of the temperature (Käs and Sackmann, 1991). In the second model, the formation of high spontaneous curvature phopholipid domains in liposome membranes generates the budding and the vesiculation of small vesicles (Lipowsky, 1993). This model, combined to the area-to-volume ratio mechanism, was supported by experiments of fluid domain formation from a gel/liquid mixed phase, after variation of the temperature (Döbereiner et al., 1993). In the third model, the budding is triggered by a change of the difference of surface area between the two elastically coupled leaflets alone (Svetina and Zeks, 1989; Miao et al., 1991; Seifert et al., 1991). According to such a model, the budding, leading sometimes to vesiculation, has effectively been experimentally triggered in response to transmembrane phospholipid translocation, as already mentioned (Farge and Devaux, 1992).

According to the first model of area-to-volume ratio changes, the plasma membrane surface area variations due to the addition of C6-PS molecules in the present experiments could effectively induce the budding and the vesiculation. However, such vesiculation is expected to be triggered immediately after the C6-PS incorporation, inducing the cell surface area-to-volume ratio perturbation. Here we observe the vesiculation rate enhancement 45 min after C6-PS addition (see Materials and Methods). This suggests that the perturbation of the plasma membrane surface area may not play a role in the present observations.

After the second model of high spontaneous curvature domain formation, budding could also be triggered, depending on the spontaneous curvature characteristics of C6-PS

and its ability to constitute segregated domains. After addition of C6-PS to the outer layer, 80% of the short-chain C6-PS is redistributed to the inner layer at steady state and may effectively form domains in the inner layer. Although the existence of PS domains in plasma membranes at 37°C still remains to be demonstrated (Moreau and Cassagne, 1994), such a mechanism could play a role in the present vesiculation observations. However, this process would require a C6-PS positive spontaneous curvature, whereas the spontaneous curvature of PS may not be positive because of its small polar head (Alberts et al., 1994) and because it is probably only slightly perturbed by the presence of a short chain of 6 carbons instead of 12.

Finally, following the third model, the present experiments interpreted in terms of the surface area asymmetry dynamical translocation alone give a correlation of $\Delta\tau/\Delta\epsilon = 2 \cdot 10^{-2} \pm 5 \cdot 10^{-3}$ between the net translocation rate and the endocytic rate. This value is in quite good quantitative agreement with the theoretical elastic prediction for τ/ϵ of $5 \cdot 10^{-2}$. Effectively, the pumping activity is also known to translocate phosphatidylethanolamine (PE) (Auland et al., 1994). Ultimately, the transport of PS and PE could be cooperative, so that the calculation of the net translocation rate increase may have been underestimated. In any case, the experimental linear correlation between the phospholipid translocation and endocytic rate suggests an elastic "pathway" for endocytosis, which depends on the transmembrane translocation activity in vivo.

Moreover, for these elastic mechanisms to be biologically relevant for endocytosis in vivo, they would need to be controlled by specific protein-dependent processes. In this context, the existence of a phospholipid translocation activity in the plasma membrane of most eukaryotic cells (for a review, see Zachowski, 1993) gives credence to the surface area asymmetry hypothesis. To our knowledge, the present experiments showing an acceleration of budding and vesiculation in vivo in response to an increase in the phospholipid translocation rate thus show for the first time the relevance of this hypothesis to living cells.

One still cannot completely exclude the hypothesis that a "static" asymmetry parameter due to the addition of PS may cause the endocytic rate increase. Because we have already ruled out the role of the spontaneous curvature asymmetry parameter, the steady-state surface area asymmetry established after PS translocation (80% of the added PS is translocated from the outer layer to the inner layer at steady state) could be such a parameter. However, experiments on giant liposomes have shown that the vesiculation is triggered as an instantaneous response to the surface area asymmetry variation, and not after its formation (Farge and Devaux, 1992). Thus, we would not expect an increase in vivo of the endocytic rate at steady-state levels, namely when the surface area asymmetry is already formed. In fact, according to this interpretation, the vesicles triggered by the translocation should already have been internalized at steady state. Instead, we observe here an increase in the endocytic rate after steady-state levels are reached.

Finally, one should bear in mind that vesiculation consists both of vesicle budding and vesicle fission. The fission may be dependent on lipid phase separation (Döbereiner et al., 1993) or on putative lipid-protein coupling processes. Unless there is no proof for PS domain formation in vivo (Moreau and Cassagne, 1994), a putative PS-protein interaction fission process in vivo could possibly trigger the fission as well as the budding in response to PS addition.

Vesiculation-fusion oscillations at short time scale

On the short time scale of seconds, membrane entry was observed to be followed by membrane exit at natural PS concentrations. These oscillations suggest that vesiculation was followed by fusion with the plasma membrane at this time scale. The oscillations are characterized by a period of typically 20 to 30 s (see Figs. 2 and 3 *A*), so that they can be considered as very rapid compared to the 10-min time scale of endocytic membrane internalization of Fig. 1 *A*. The existence of very rapid recycling vesiculation-fusion processes has previously been proposed (Subtil et al., 1995; Mayor et al., 1993; Steinman et al., 1983). We here observe direct evidence of such a dynamic process in plasma membrane, for the first time to our knowledge.

Moreover, the short time scale vesiculation and fusion rates are experimentally found to be dependent on the same translocation activity. Effectively, the vesiculation and fusion rates are found to be proportional to the translocation rate. Consequently, the two dynamics can be considered to be coupled by the same phospholipid translocation process. This coupling presents the plasma membrane as a dynamic structure, oscillating between two distinct curvature states, one of 10^{-1} μm^{-1} (the normal state) and the other of 10 μm^{-1} (the vesiculated state).

The translocation-induced fusion may be understood within the context of recent scaling theoretical considerations describing the elastic reponse of closed bilayers to phospholipid translocation (Farge, 1994; Farge and Devaux, 1993). This response was proposed to be dependent on the scale, leading to vesiculation in closed bilayers of the size of cell plasma membranes, but to net dilation in membranes as small as endocytic vesicles. It was proposed that such a dilation effect may favor fusion by opening lipid or peptide pores (Farge, 1994). Likewise, a putative cotransport of PS and PE may increase the concentration of PS and PE (fusogenic lipids; Moreau and Cassagne, 1994) in the outer layer of the vesicles in reponse to PS translocation acceleration.

We must note that vesiculation at short time tail is 10 times too rapid to be interpreted in terms of the equilibrium elastic model theory of vesicle stabilization, and that it may involve a process of unstable spontaneous vesiculation (Bruinsma, 1990; Nezil et al., 1992). On the other hand, stabilization of vesiculation resulting from the balance between the rapid vesiculation and the fusion of $\Delta\tau/\Delta\epsilon = 1.5 \cdot 10^{-2} \pm 10^{-3}$ is shown to be on the comparable to the equilibrium elastic prediction of $5 \cdot 10^{-2}$.

CONCLUSION

The molecular basis of membrane invagination and vesicle formation during the initial steps of endocysis still remains to be fully elucidated for living cells (Mellman, 1994). We show here in vivo that phospholipid translocation may generate a surface area asymmetry resulting in vesiculation. Thus, endocytic vesiculation is a physiological process that may be considered to be generated in reponse to the interaction between the protein-dependent phospholipid translocation activity and the elastic properties of plasma membranes. Within this model, the role of proteins like clathrin would be to regulate the specificity of endocytosis (Rothman, 1994), instead of inducing vesiculation (Jin and Nossal, 1993). Experiments showing that clathrin-coated pits are not necessarely required for vesiculation effectively support such a model (Cupers et al., 1994; Subtil et al., 1995).

In addition, the plasma membrane is proposed to be a highly dynamic structure of rapid vesiculation and refusion (Subtil et al., 1995). Endocytosis (membrane internalization) and exocytosis (membrane exit) may thus appear as a unified process of membrane oscillation, possibly controlled by a translocation activity (Farge, 1994). Such a proposal may also explain observations of oscillations occurring during the exocytosis process of synaptic vesicle secretion, where the vesicles were found to be mechanicaly strain-dilated before the fusion event (Monck et al., 1990). Such an oscillation may generally act as a reservoir for the endo-exocytic vesicles observed under the cytosolic leaflet of the plasma membrane.

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