Endocytosis Switch Controlled by Transmembrane Osmotic Pressure and Phospholipid Number Asymmetry

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ABSTRACT The dynamics of endocytosis in living K562 cells was investigated after the osmotic pressure of the external medium was decreased and the transmembrane phospholipid number asymmetry was increased. When the external pressure was decreased by a factor of 0.54, a sudden inhibition of endocytosis was observed. Under these conditions, the endocytosis suddenly recovered after the phospholipid number asymmetry was increased. The phospholipid asymmetry was generated by the addition of exogenous phosphatidylserine, which is translocated by the endogenous flippase activity to the inner layer of the membrane. The recovery of endocytosis is thus consistent with the view that the phospholipid number asymmetry can act as a budding force for endocytosis. Moreover, we quantitatively predict both the inhibition and recovery of endocytosis as first-order phase transitions, using a general model that assumes the existence of a transmembrane surface tension asymmetry as the budding driving force. In this model, the tension asymmetry is considered to be elastically generated by the activity of phospholipid pumping. We finally propose that cells may trigger genetic transcription responses after the internalization of cytokine-receptor complexes, which could be controlled by variations in the cytosolic or external pressure.

INTRODUCTION

Endocytosis is initiated by the budding of ~70-nm-diameter vesicles from living cell plasma membranes (Alberts et al., 1994). It is a ubiquitous cell process that regulates the internalization of external protein as well as of plasma membrane proteins. Endocytosis is important for the function of eukaryotic cells. For instance, external signaling protein cytokines are known to trigger cell genetic transcriptions, once they are linked to their specific membrane receptor (Yamamoto et al., 1997). The endocytic internalization of the cytokine-receptor complex into the cell is generally thought to inhibit the transcription response, after a "degradation" of the cytokine-receptor interaction into the acidic cytosolic compartments (like endosomes). But, depending on the cytokine, it may, on the contrary, activate the signal transduction leading to the genetic transcription (Baskin et al., 1991; Koenig and Edwardson, 1997). Therefore, the modulation of endocytosis is a potentially important regulator of cytokine-dependent cell genetic transcription. However, the origin of the forces initiating as well as the forces modulating endocytic vesiculation still remains one of the debated questions of the cell biology (Maddox, 1993; Cupers et al., 1994; Matsuoka et al., 1998).

So far, two biochemical activities have been found experimentally to be involved as driving forces of the vesicle formation in vivo. The first one is the polymerization of proteins like clathrin, or calveole, onto the cytoplasmic phospholipid leaflet. Such polymerization is thought to locally force the curvature of the membrane (Rothman, 1994; Jin and Nossal, 1993; Mashl and Bruinsma, 1998). The second activity involves the active generation of a phospholipid number asymmetry between the two monolayers of the plasma membrane. Such asymmetry is thought to elastically generate the plasma membrane curvature necessary to trigger the budding of small endocytic vesicles (Farge and Devaux, 1992; Farge, 1994). The latter process was shown to use the ubiquitous activity of phospholipid pumping, which specifically translocates aminophospholipids from the outer to the inner layer of the plasma membrane (Seigneuret and Devaux, 1984; Tang et al., 1996). Effectively, it has been shown that the addition to the outer layer of specific aminophospholipids, which are actively translocated to the inner layer by pumping activity, leads to an increase in the endocytic dynamic from a factor 2 to 4, in living K562 cells (Farge, 1995; Farge et al., 1999).

Here, the external osmotic pressure is characterized as a critical force of sudden "on-and-off" endocytosis modulation. The budding is investigated theoretically, as well as experimentally in vivo, in response to a decrease in the external osmotic pressure.

First, we propose theoretically the existence of a difference in surface tension $\Delta\sigma = \sigma_1 - \sigma_2 > 0$ between the inner and the outer monolayers of the plasma membrane (denoted as 1 and 2) as the driving force of endocytic vesiculation. We here assume $\Delta\sigma$ to be mechanically generated by the phospholipid number asymmetry between the two monolayers of the plasma membrane, ΔN, generated continuously by the translocation activity. Second, radically different from a simple liposome, the plasma membrane of the living cell is here considered to be in contact with a dynamic reservoir of surface area, due to the endocytic vesicles that continuously recycle from internal compartments. Following these two assumptions, we investigate the

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conditions of local equilibrium associated with the existence of a bud still connected to the plasma membrane. We find that the model of transmembrane tension asymmetry generically predicts the budding as a membrane instability, that is, that the plasma membrane flows spontaneously into small-radius structures. Moreover, it predicts a sudden inhibition of the budding process, described as a first-order phase transition, in response to the application of a pressure asymmetry, $\Delta p = p_1 - p_2 > 0$, applied between the inner and outer cell volumes (1) and (2), respectively. The transition results from the competition between the two antagonistic forces applied to the bud membrane: the Δp volumic asymmetry force that opposes the vesiculation, and the $\Delta\sigma$ membrane tension asymmetry, the driving force of the budding. Following this prediction, the transition should trigger a sudden inhibition of cell endocytosis in vivo.

The existence of such a transition is here experimentally observed in living cells, by decreasing the external pressure through successive dilution, with distilled water, of the external medium. The experimental critical pressure asymmetry Δp_c at transition quantitatively correlates with the predicted value. Moreover, at high pressure asymmetry ($\Delta p > \Delta p_c$), we observe a reverse transition of sudden endocytosis recovery in response to the increase in the phospholipid number asymmetry. The phospholipid number asymmetry is increased after the addition to the outer layer, and the active translocation to the inner layer, of exogenously added phosphatidylserine, an aminophospholipid specifically pumped by the translocation activity. The reverse transition is predicted by the model. The experimental value of the critical phospholipid number asymmetry increase at transition, ΔN_c, also correlates with the theoretical prediction.

First, these experimental results suggest that the phospholipid number asymmetry can be considered to be an endocytic budding driving force without clathrin in terms of the generation of a steady-state membrane tension asymmetry. Second, we here experimentally demonstrate, in living cells, that the osmotic pressure is a critical force of endocytosis modulation, in quantitative agreement with predictions of soft-matter physics. The latter could be exploited by cells to accurately control genetic transcriptions that depend on the endocytosis of cytokine signaling molecules.

THEORY

At equilibrium, the plasma membrane is characterized by a membrane tension asymmetry $\Delta\sigma = \sigma_2 - \sigma_1$, where σ_2 and σ_1 are, respectively, the outer and inner layer membrane tensions. We assume that the inner layer is "compressed" ($\sigma_1 < 0$) and the outer layer is "dilated" ($\sigma_2 > 0$), so that $\Delta\sigma > 0$. Moreover, the cell is characterized by the osmotic pressure asymmetry $\Delta p = p_2 - p_1$, where p_2 and p_1 are, respectively, the outer and inner medium pressures. We assume a decrease in the external medium osmotic pressure p_2, so that $\Delta p > 0$. For a given value of Δp, the global shape of the plasma membrane is kept constant and quasispheric. This is due to the presence of the cell cytoskeleton or to the cytosolic water incompressibility. As a consequence, the constraint $\Delta\sigma$ can exclusively relax locally, through the liquid plasma membrane flow into small-radius buds. We thus describe the vesiculation as a liquid surface area exchange between the plasma membrane and the connected spherical cap bud of radius R and neutral surface area A_0 (see Fig. 1 *A*). Thus R and A_0 are two independent variables. (The bud is considered as a spherical cap, because this shape a priori minimizes the membrane bending energy associated with the local curvature, at a constant surface area A_0. Moreover, following the method of Lipowsky (1993), and for the sake of simplicity, the energy of the membrane that connects the bud to the plasma membrane is not taken into consideration.) In addition, we consider the plasma membrane to be a surface area reservoir for the bud (see just below). The bud is thus characterized by the membrane tension asymmetry $\Delta\sigma$ of the plasma membrane to which it is connected. In the presence of a pressure asymmetry, three energy terms describe the bud generation from the plasma membrane.

The first term is the budding driving force. It is the energy due to the relaxation of the membrane tension asymmetry of the plasma membrane, with the inner layer surface area increase and the outer layer surface area decrease associated with bud formation. At first order in h/R, the energy can be written as $\Phi_1 = -(h/2R)\Delta\sigma A_0$, where h is the membrane thickness (see the legend to Fig. 1 *A*). Importantly, the cell plasma membrane mean tension σ generated by the pressure asymmetry plays no role in Φ_1. This assumption is a consequence of the existence of an endocytic vesicle recycling process in living cells. Effectively, exocytic vesicles continuously bud from the internal cytosolic membranes and fuse with the plasma membrane. At steady state, the mean phospholipid number of the plasma membrane lost into one vesiculation is compensated for, at the same time, by the increase in the phospholipid number associated with one vesicle fusion. This recycling process is well known to maintain the entire surface area of cells in vivo (Steinman et al., 1983). Therefore, the mean phospholipid number remains constant during vesiculation. This implies that no elastic dilation of the plasma membrane is needed to give rise to the phospholipid number necessary for the vesicle formation. As a consequence, no variation in mean surface tension is needed to generate the bud. Thus the membrane tension σ is not involved in the energy that characterizes bud generation. (We here implicitly assume that, at equilibrium, the cytosolic membranes and the plasma membrane are necessarily characterized by the same mean membrane tension.) It remains constant during the vesiculation, which is the definition of a surface area reservoir. Therefore, even a plasma membrane that is stretched should vesiculate,

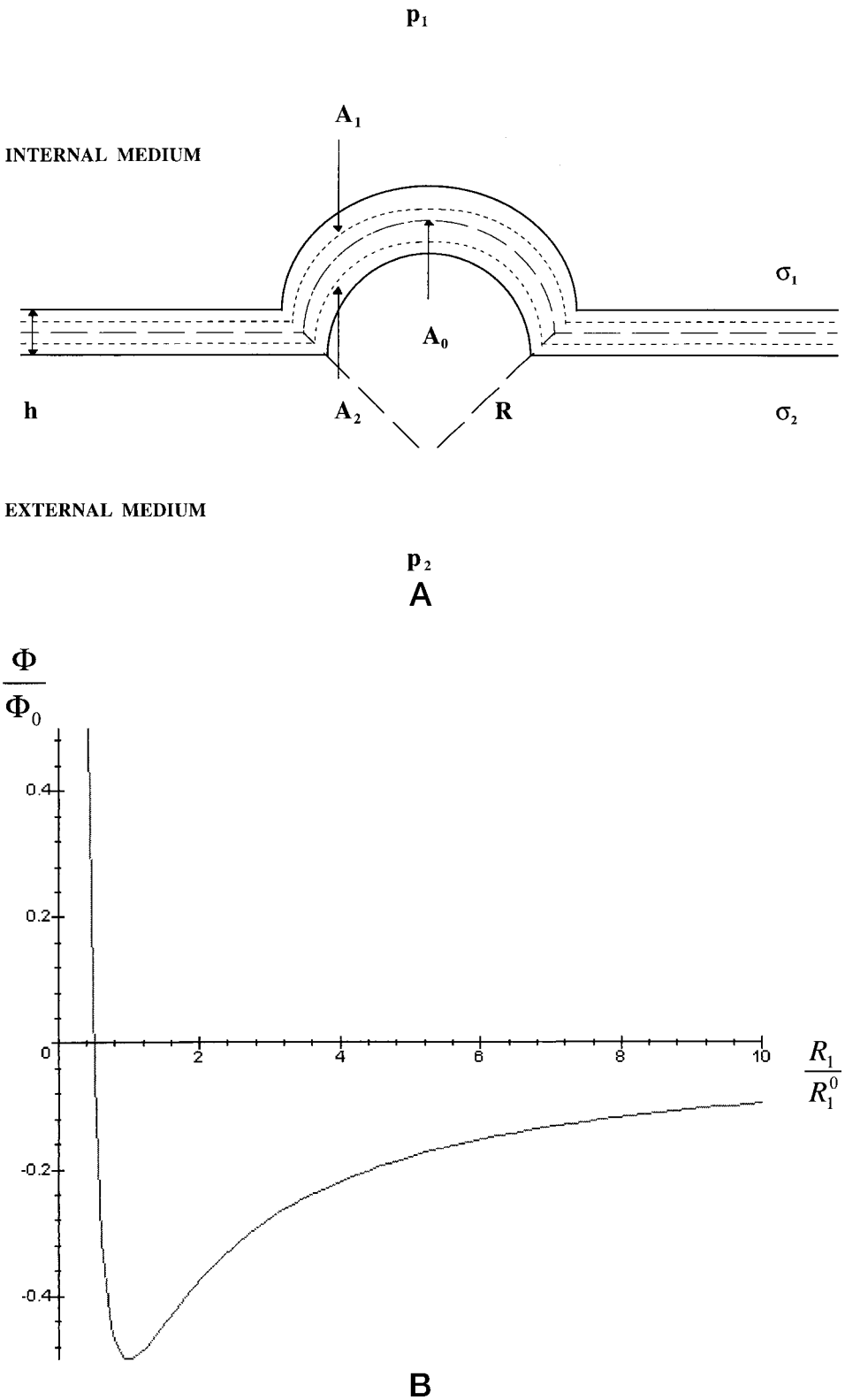

FIGURE 1 (*A*) The bud as a liquid membrane spherical cap, connected to the plasma membrane. R is the bud radius, A_0 its neutral surface area, and h the membrane thickness. p_2 and p_1 are the outer and inner volumic pressures. A_2 and A_1 are the neutral surface areas of the outer and inner monolayers, respectively ($A_2 = A_0(1 - (h/2R))$ and $A_1 = A_0(1 + (h/2R))$), at first order in h/R. σ_2 and σ_1 are, respectively, the outer monolayer and inner monolayer tensions of the plasma membrane. The budding driving force energy Φ_1 is associated with the partial relaxation of the plasma membrane tension asymmetry $\Delta\sigma$, through the increase in the inner layer surface area, $\delta A_1 = A_0(h/2R)$, and the decrease in the outer layer surface area, $\delta A_2 = -A_0(h/2R)$, both of which are associated with the local plasma membrane curvature $1/R$ generated by the bud formation. This can be written as $\Phi_1 = \sigma_1\delta A_1 + \sigma_2\delta A_2 = -(h/2R)\Delta\sigma \cdot A_0$. (*B*) The potential $\Phi(R; A_0)$ of the bud at $\Delta p = 0$, normalized to $\Phi_0 = 4\pi KhR_1^0$, as a function of R. The competition between the membrane tension asymmetry $\Delta\sigma$ and the monolayer bending energy generates a first equilibrium radius $R_1^0 = 16k_c/h\Delta\sigma$ (see text). It is easy to see by the figure that $(\partial\phi/\partial A_0)|_{R=R_{01}} \leq 0$, because $\Phi(R_1^0; A_0) \leq 0$, as Φ is homothetic to A_0. The exact calculation effectively leads to $(\partial\phi/\partial A_0)|_{R=R_{01}} = -(1/4^3)((h\Delta\sigma)^2/k_c) \leq 0$. As a consequence, the equilibrium radius R_1^0 spontaneously recruits surface area from the liquid plasma membrane. R_1^0 is thus the length scale of the vesiculation. K is the elastic modulus of dilation of a monolayer.

because of the plasma membrane contact with a reservoir of internal cytosolic membranes.

The second term is the energy of bending of both monolayers that opposes budding: $\Phi_2 = 4k_c A_0/R^2$, where k_c is the bending elastic constant of a monolayer (Helfrich, 1973). Note that we only formulated the bulk-flow endocytic vesiculation, which is clathrin independent. The energy associated with clathrin polymerization is thus not taken into consideration.

Moreover, plasma membrane monolayers are here assumed to be characterized by a zero spontaneous curvature.

From the competition between Φ_1 and Φ_2, we deduce a first equilibrium radius R_1^0: $R_1^0 = 16k_c/h\Delta\sigma$. Moreover, we find $(\partial\phi/\partial A_0)|_{R=R_1^0} \leq 0$ for $\Phi = \Phi_1 + \Phi_2$ (see Fig. 1 *B*). We thus show the bud as unstable with regard to the variable A_0 at R_1^0, because R_1^0 spontaneously recruits surface area from the liquid plasma membrane. R_1^0 is therefore the budding vesiculation length scale. As a consequence, R_1^0 should take plasma membrane surface area without limitation, leading to long membrane tubular structures. (These dynamic structures grow into a viscous medium at low Reynold's number (Purcell, 1977), so that the flow should minimize the energy of hydrodynamic dissipation (Acheson, 1991). Compared with the tubular structures, the pearl shape adds a curvature to the velocity field, which induces an upper dissipation of hydrodynamic energy. The dynamical tubular structure should thus be selected instead of the static pearl-shaped structure.) Interestingly, this is what is effectively observed in vivo, if the dynamin protein that triggers the fission of newly formed vesicles is not present in dynamin *Drosophila* mutants (Urrutia et al., 1997).

The third term is the bud volumic energy: $\Phi_3 = -\gamma RA_0\Delta p$. This expression is valid around the closed state of the vesicle, with $\gamma = 1/3$, which is the state we are interested in. We specifically ask whether the external osmotic pressure conditions allow the formation of a complete vesicle. From the competition between Φ_1 and Φ_3, a second equilibrium radius is deduced that is unstable with regard to the variable R (see Fig. 2 *A*): $R_2 = (3h\Delta\sigma/2\Delta p)^{1/2}$. From Fig. 2 *A*, we see that the vesiculation solution R_1 collapses at $R_1 = R_2$, namely at the critical pressure asymmetry $\Delta p_c \propto (h\Delta\sigma)^3/(16k_c)^2$. The exact expression can be written as $\Delta p_c = (2/3^2)\ (h\Delta\sigma)^3/(16k_c)^2$ for a critical budding radius $R_c = (3/2)R_1^0$ (see the legend to Fig. 2).

Therefore, from the model involving the membrane tension asymmetry as a budding driving force emerges a first-order phase transition leading to the inhibition of the vesiculation under hypoosmotic constraints. This is predicted whatever the origin of $\Delta\sigma$.

In our case, we propose that the membrane tension asymmetry is generated by the existence of phospholipid number asymmetry between the two layers of the plasma membrane, ΔN, continuously generated by the phospholipid pumping activity. From Hooke's law, the mechanical tension asymmetry of the membrane is written as $\Delta\sigma = K(\Delta N/N_m)$, where K is the modulus of elastical dilation of a monolayer of the plasma membrane and N_m is the mean phospholipid number of the plasma membrane.

MATERIALS AND METHODS

Cells and materials

K562, a human erythroleukemia cell line, was grown in suspension in RPMI 1640, 10% decomplemented fetal calf serum, supplemented with 2 mM L-glutamine. The short-chain phosphatidylserine, with six carbons on the second chain (C6-PS) (a generous gift from Paulette Hervé, IBPC, Paris), was synthesized by following a previously described protocol (Fellman et al., 1994), and lyso-α-phosphatidylserine (1-PS) was purchased from Sigma (St. Louis, MO). *N*-Hydroxysuccinimidobiotin (NHS-biotin) and streptavidin-fluorescein isothiocyanate were purchased from Pierce (Rockford, IL).

FITC-streptavidin-biotin labeling of surface membrane proteins

After the cells were incubated for 30 min with or without exogenous lipid and the mixture was cooled to 0°C, 4 mg/ml of NHS-biotin was added for 30 min on ice. The cells were then washed three times in 1 ml phosphate-buffered saline (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 finally resuspended in a final volume of 350 μl PBS on ice.

Endocytosis monitored by spectrofluorimetry

The fluorescein isothiocyanate (FITC) moiety was used to follow endocytosis due to the pH sensitivity of its fluorescence. Thus the fluorescence is quenched when FITC is transferred from the extracellular pH of 7.4 to the more acidic environment of an endocytic compartment (Sorkin et al., 1988; Carraway and Cerione, 1993). Hence the fluorescence of the FITC-labeled proteins decreases as a function of time because of their internalization into endocytic compartments.

All measurements were performed with an ISA-SPEX (Instruments SA Group) spectrofluorimeter. Three hundred and fifty microliters of the FITC-labeled K562 cells suspended on ice was transferred directly into the spectrofluorimeter cuvette, which was previously kept at 37°C. One milliliter of PBS at 60°C was gently added to the cuvette by drops over 20 s while mixing to reach a final temperature of 37°C at thermal equilibrium. The suspension was excited at $\lambda_{ex} = 488$ nm, and the fluorescence intensity was measured at $\lambda_{em} = 508$ nm as a function of time, with points taken every 10 s during the entire experiment, which typically lasted 15 min. The relative fluorescence quenching at time t was defined as $1 - [F(t)/F(0)]$, where $F(0)$ is the fluorescence at $t = 0$. The kinetics of the fluorescence quenching were measured separately as a function of the dilution, with distilled water, of the external medium, and of the concentration of exogenous lipid that had been previously added to the K562 cells. Initial slopes were determined, assuming an exponential form for the dynamics of fluorescence quenching $F(t)$, following $F(t) = I + I_0 \exp(-t/t_0)$, where $F(0) = I + I_0$ is the initial fluorescence intensity and I_0 is the amplitude of the fluorescence quenching. I and I_0 are measured at steady state, after the saturation of the dynamics of fluorescence quenching. From $\log(F(t) - I)$, we extract the characteristic time t_0. The initial slope (percentage of plasma membrane internalized per time unit) is finally deduced from $\alpha = -I_0/F(0)(1/t_0)$.

Control of the external osmotic pressure

The external osmotic pressure was modified by successive dilutions of the isotonic PBS medium with distilled water at time zero of the endocytic measurements, directly into the spectrofluorimeter cuvette previously kept at 37°C with a thermostat. The temperature of the added distilled water was systematically adjusted to reach a 37°C solution just after mixing.

Preincubation of cells with C6-PS and lyso-PS

After centrifugation at 1200 rpm for 8 min in culture medium, 50 μl of K562 cell pellet was washed in 50 ml of PBS and resuspended in 100 μl

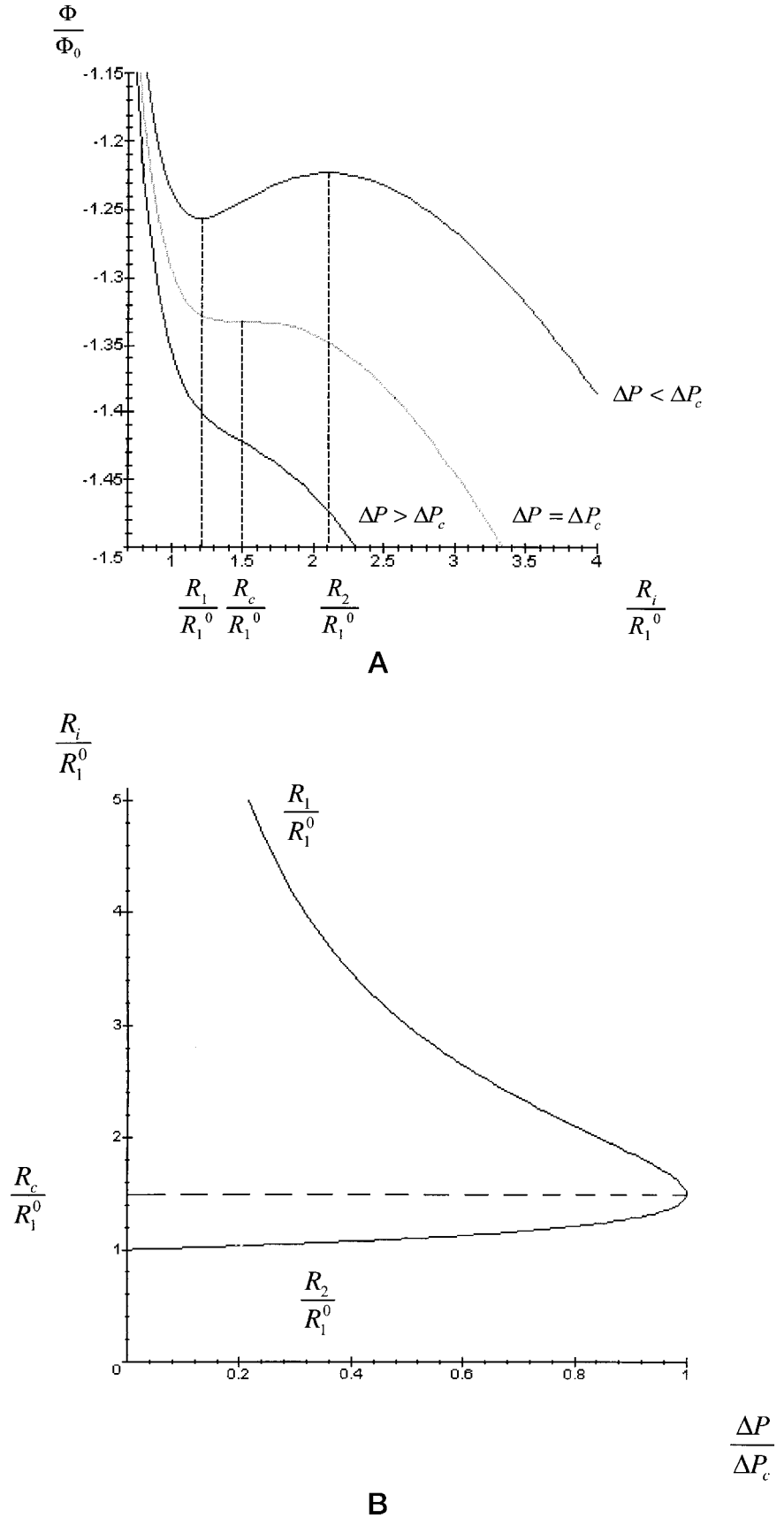

FIGURE 2 (*A*) The potential $\Phi(R; A_0, \Delta p)$ of the bud normalized by $\Phi_0 = 4\pi K h R_1^0$ as a function of R, for different values of Δp. The competition between the energies of the membrane tension asymmetry $\Delta\sigma$ and the pressure asymmetry Δp generates a second equilibrium radius $R_2 = (3h\Delta\sigma/2\Delta p)^{1/2}$ (see text), which is unstable. R_1 and R_2 collapse at $(\partial\phi/\partial R)|_{A_0} = 0$ and $(\partial^2\phi/\partial R^2)|_{A_0} = 0$, namely at the critical pressure asymmetry $\Delta p_c = (2/3^2)\ (h\Delta\sigma)^3/(16k_c)^2$ and at the critical radius $R_c = (3/2)R_1^0$. There are no further vesiculation solutions for $\Delta p > \Delta p_c$, which describes a first-order phase transition of vesiculation inhibition at Δp_c. (*B*) The equilibrium radii of the bud R_1 and R_2 normalized by $R_1^0 = R_1$ ($\Delta p = 0$), as a function of Δp. From $(\partial\phi/\partial R)|_{A_0} = 0$ and from Montferrier (1837) the exact analytical solutions for R_1 and R_2 plotted on the figure are deduced (details of the calculation are not shown). Note that R_1 varies only slightly with Δp, and that we effectively have $R_c = (3/2)R_1^0$.

of PBS. C6-PS (3.75–22.5 nmol) was dried on glass under nitrogen flow and then directly resuspended in 100 μl of PBS; lyso-PS was used at 11.25 nM and 22.5 nM in PBS. The percentage of added lipids was previously evaluated by measuring the linewidth broadening due to spin-spin interactions (Farge, 1995) for plasma membrane concentrations that are typically 1% spin-labeled phospholipid (Marsh and Smith, 1973). The experiment consists of adding spin-labeled analogs to the 50 μl resuspended in 100 μl PBS and determining the quantity of added analogs at which the linewidth broadens. This quantity, found to be 7.5 nmol, represents a concentration of 1% of the lipid in plasma membranes. The solution was then subjected to ultrasonication for 1 min to completely solubilize the lipid. This solution was added directly to the cell suspension (10^5 cells/ml). At these low concentrations, C6-PS or lyso-PS is incorporated spontaneously into the outer layer of the plasma membrane bilayer (Cribier et al., 1993). C6-PS, but not lyso-PS, is translocated by the flippase to the inner layer after 30 min of incubation at 37°C (Cribier et al., 1993; Zachowski, 1993). The cells were then cooled to 0°C to stop any endocytic activity before proceeding with labeling of the outer layer surface proteins of the cells. Transmembrane transport of the aminophospholipids was controlled using spin-labeled analogs, as described by Cribier et al. (1993). After incubation with the above lipids, the viability of the cells was determined by trypan blue exclusion after incubation of the cells under the same conditions as used for fluorescence measurements.

Quantification of the C6-PS translocated onto the inner layer of the plasma membrane after 30 min of incubation at 37°C

The C6-PS translocation in K562 cells was monitored by following a previously described protocol (Cribier et al., 1993), by adding 0.5–3% of the C6-NBD-PS fluorescently labeled analog to the outer layer of the plasma membrane. The translocation quantification was measured from the amount of NBD-label probe left on the outside membrane after 30 min of incubation at 37°C, compared to the NBD-label probe quantity initially added. These quantities were measured by bovine serum albumin (BSA) extraction of the outer layer probe fraction that had not been translocated. Briefly, after the addition of the probes to the outer layer of the plasma membrane at time zero, 50-μl aliquots were taken from the cell solution at 30 min. They were added to 120 μl of a 1% (w/v) BSA solution in PBS medium maintained at 4°C. After 45 s at 4°C, any probe that had bound to the BSA is separated from the cells by centrifugation for 30 s at 7600 $\times$ g. The quantity of initially added NBD-label probes was measured by following the same protocol, but without the cells. The fraction of probe that was not internalized was finally obtained by measuring the fluorescence intensity of the BSA/NBD-label complexes in solution in the spectrofluorimeter.

RESULTS

The endocytic vesiculation dynamics of the K562 living cells (a cell line classically used for biological endocytosis studies) was first measured as a function of the decrease in the external osmotic pressure. The external osmotic pressure was simply controlled by successive dilutions of the external isotonic medium PBS with distilled water, at time zero of the endocytosis measurement. The dilution factor is denoted by ϕ ($\phi = 1$ means no dilution). To measure the dynamics of the internalization of the plasma membrane by endocytic pathways, we followed the method of Farge et al. (1999). The plasma membrane proteins were fluorescently labeled with FITC, using the FITC-streptavidin-biotin complex. The principle of the method is based on the high pH-dependent sensitivity of the FITC fluorescence intensity. Once internalized by endocytosis into the pH 5–pH 6 acidic internal cytosolic compartments, the FITC intensity is quenched at 75%, compared with its intensity in the external medium at pH 7.4 (Sorkin et al., 1988). As a consequence, the decrease in the FITC signal monitors the dynamics of endocytosis of FITC-labeled plasma membrane proteins (Carraway and Cerione, 1993). Experimental procedures are described in detail in the Materials and Methods section. Note that the protein internalization through the clathrin pathway represents ~2% of the plasma membrane protein endocytic traffic only (Dautry-Varsat and Lodish, 1984). In the present experiment, plasma membrane proteins are statistically labeled using a biotin-streptavidin complex. As a consequence, only 2% of the signal should be attributed to the clathrin pathway. Thus we here exclusively measure the bulk-flow endocytosis dynamics, in agreement with the theoretical model, whereby clathrin polymerization is not taken into consideration.

Fig. 3 *A* shows the dynamics of internalization of the plasma membrane by endocytic pathways, at different external medium dilutions ϕ. A decrease in dynamics is observed in response to the decrease in ϕ, with a sudden variation between $\phi = 0.8$ and $\phi = 0.4$. The experiment was performed for several values of ϕ, each time doubled by its control experiment at $\phi = 1$. The initial slope α of each experiment was normalized to the initial slope α_0 of its control and plotted as a function of $1 - \phi$ in Fig. 3 *B*. A linear decrease in the endocytic dynamics is observed from $\alpha/\alpha_0 = 1$ to $\alpha/\alpha_0 = 0.65$ between $\phi = 1$ and $\phi = 0.54$. A sudden transition, leading to a nearly complete inhibition of the endocytic internalization, is observed at the critical dilution value $\phi_c = 0.54 \pm 0.1$. The blue trypan viability test revealed that the cells stayed alive until dilution $\phi = 0.2$. The cells were effectively preincubated for a few minutes with 0.18% trypan blue at 37°C, before the osmotic constraint was applied. The same thing was done at the end of the experiment. No transient trypan blue internalization was observed during the hypotonic shock. No blue trypan accumulation in cells was observed on the time scale of the experiments (not shown). The existence of a sudden transition leading to the endocytic vesiculation inhibition, in response to the decrease in the external medium pressure, qualitatively confirms the prediction related to the model of plasma membrane tension asymmetry for endocytic vesiculation.

We now test the hypothesis that the observed transition may involve a mechanical membrane tension asymmetry $\Delta\sigma$ that could specifically be generated by the phospholipid number asymmetry, due to the phospholipid translocation activity. Experiments were performed at the fixed dilution value $\phi = 0.2$ (far into the inhibition regime), progressively increasing the phospholipid number asymmetry of the plasma membrane, ΔN. The phospholipid number asymme-

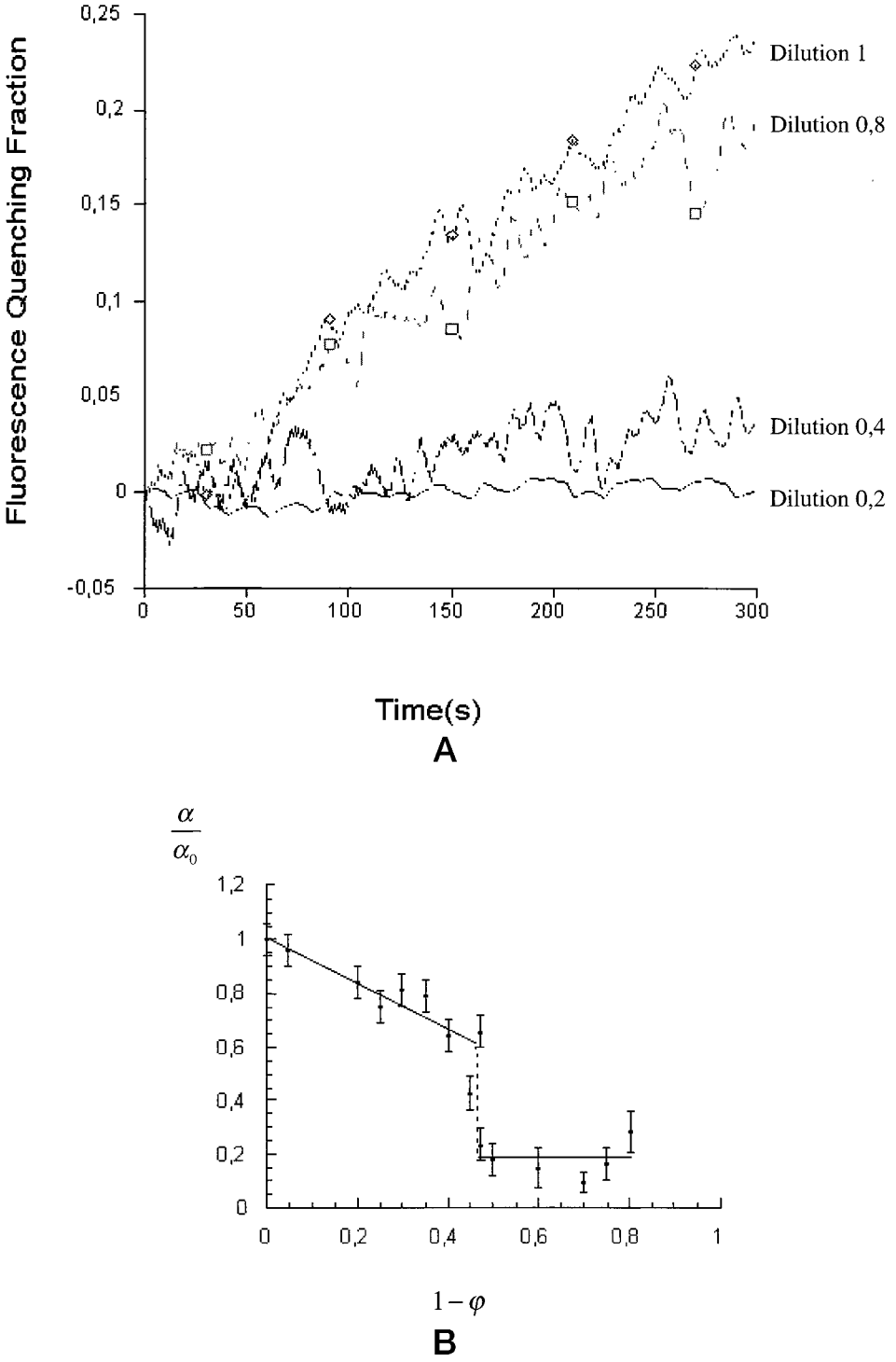

FIGURE 3 (*A*) The early stage of the endocytic dynamics as a function of the external medium dilution ϕ. The decrease in the external osmotic pressure induces a decrease in the endocytic internalization rate, with an important gap observed between dilutions $\phi = 0.8$ and $\phi = 0.4$. (*B*) The endocytic internalization rate as a function of the dilution factor ϕ. The initial slope α of the endocytic internalization dynamics under hypoosmotic constraints, normalized by the experimental initial slope α_0 for $\phi = 1$, is plotted as a function of $1 - \phi$. A sudden inhibition of endocytosis is observed at $\phi_c = 0.54$, after a linear decrease in the endocytosis dynamics. Each point represents two experiments, except at the transition, where one point is one experiment.

try increase was generated by adding δN exogenous C6-PS phospholipids to the outer layer, which were actively translocated onto the inner layer within 30 min (Cribier et al., 1993). (Note that we assume here that the phospholipid number asymmetry generated by the translocation is not lost by the vesiculation on the 30-min time scale. The validity of this hypothesis is supported by the rapid recycling process of endocytic vesicles from internal compartments to the plasma membrane, which acts on the 10-min time scale. This recycling process ensures the conservation of the plasma membrane surface area, whatever the endocytic rate. It should conserve the phospholipid number asymmetry of the plasma membrane as well, which is also recycled by the endocytic vesicles.)

Note that we verified that almost all of the C6-PS added to the outer layer was translocated after 30 min of incubation at 37°C. We used C6-NBD-PS, a fluorescently labeled C6-PS analog. In the range of the C6-PS concentration used in the experiment, we found that the percentage of translocation remains constant at ~90% of the newly added phosphatidylserine (see Fig. 4). Moreover, we verified that the translocation of additional C6-PS is not accompanied by an active transfer of native PS from the inner to the outer layer. We incubated cells for 30 min at 37°C with 1% C6-NBD-PS analogs and for 30 more min with 1% C6-PS at 37°C. We found a negligible transfer from the inner to the outer layer of 2% of the C6-NBD-PS only. This showed no putative "floppase" activation (Kamp and Haest, 1998) that was able to counterbalance the flippase activity resulting from the addition and translocation of C6-PS. Because the active pathway for flippase-floppase activities are identical for PS and the phosphatidylethanolamine PE, this result predicts the same behaviour for PE. No balancing effect is expected for other lipids that passively cross membranes on much larger time scales.

The osmotic constraint was applied at time zero of the endocytosis measurement, after the establishment of the phospholipid number asymmetry between the two monolayers of the plasma membrane (see Materials and Methods). A recovery of the endocytic dynamics is observed in response to the addition and translocation of δN molecules, between $\delta N/N_m = 1.5\%$ and $\delta N/N_m = 2.5\%$ (see Fig. 5 *A*). A sudden transition, leading to the endocytosis recovery, is observed at the critical value of the phospholipid number asymmetry $\delta N_c/N_m = 2\%$ (see Fig. 5 *B*). The blue trypan viability test revealed cells to be alive at a dilution of $\phi = 0.2$, up to 3% of C6-PS added and translocated. No transient blue trypan internalization was observed during the osmotic shock (not shown). Importantly, adding to the outer layer 3–6% of lyso-PS, a single-chain phospholipid that is closely related to C6-PS but is not recognized by the translocation activity (Zachowski, 1993), did not trigger the vesiculation recovery transition. This shows the necessity of the phospholipid translocation to the inner layer to trigger the recovery transition. The existence of a sudden reverse transition of endocytosis recovery, triggered by the increase in the asymmetrical number of phospholipids at high dilution ($\phi < \phi_c$), qualitatively suggests the existence of membrane tension asymmetry $\Delta\sigma$ as a driving force of the vesiculation, generated by the coupling of the pumping activity and the elastic properties of the plasma membrane.

COMPARISON BETWEEN THEORY AND EXPERIMENTS

To compare the critical pressure asymmetry at transition, deduced from experiments, with the value predicted from theoretical analysis, we used Laplace's Law applied to the surface area of the plasma membrane. Thus Δp can be written as $\Delta p = 2\sigma/R_m$, where σ and R_m are, respectively, the mean elastic membrane tension and the radius of the plasma membrane.

Transition of endocytic vesiculation inhibition

Initially, K562 cells have a radius of $R_{0m} = 10\ \mu m$. At transition ($\phi_c = 0.54$), we measured a mean cell radius R_{cm}

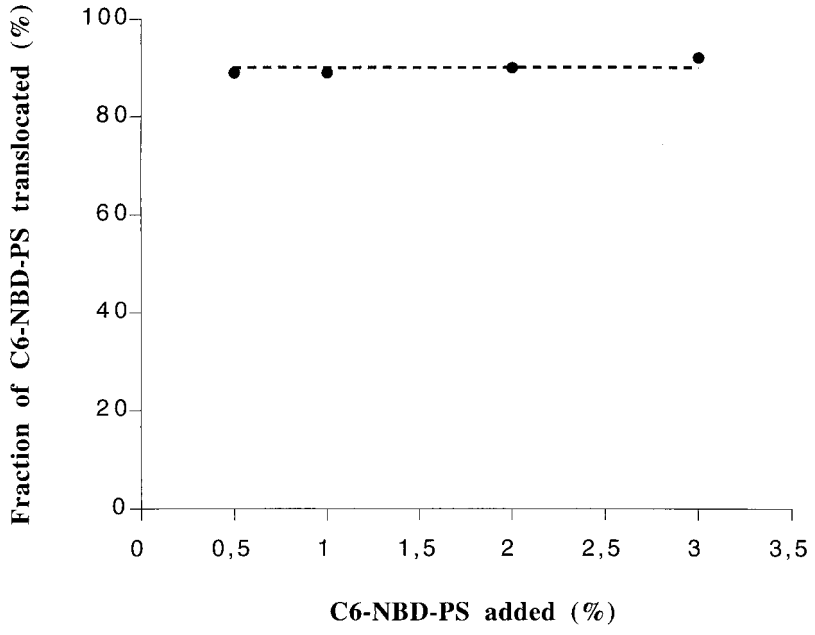

FIGURE 4 Quantification of the C6-NBD-PS fraction translocated to the inner layer after 30 min of incubation at 37°C, as a function of the C6-NBD-PS concentration added to the outer layer. C6-NBD-PS is a fluorescently labeled analog of C6-PS. In the concentration range of the experiments, namely from 0.5% to 3% of C6-PS increase, ~90% of the added C6-NBD-PS analog is translocated to the inner layer, after 30 min of incubation at 37°C. Almost all of the added phosphatidylserine is thus translocated, with no detectable saturation effect.

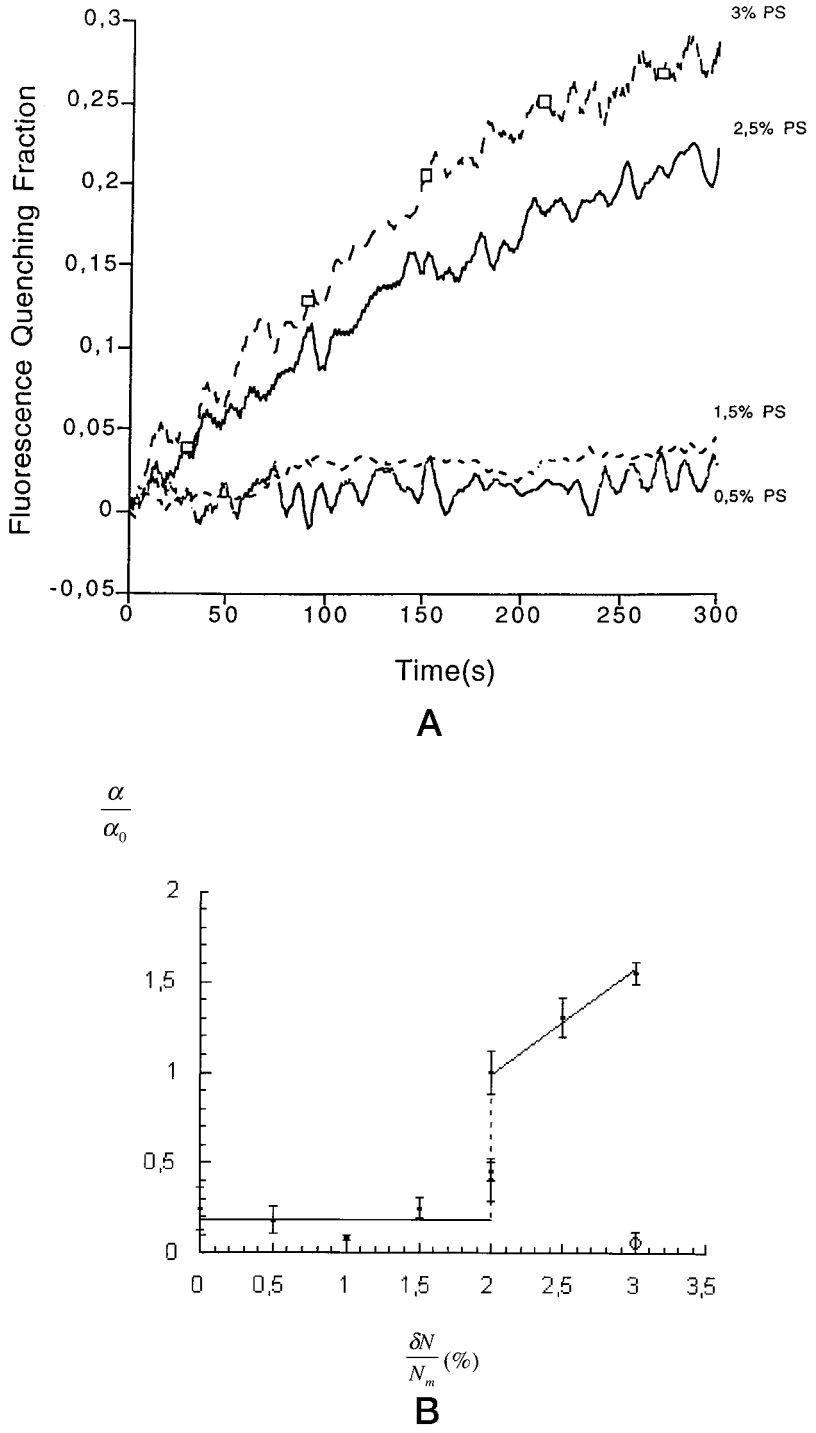

FIGURE 5 (*A*) The early stage of the endocytic dynamics at high hypoosmotic constraint $\phi = 0.2$, as a function of the percentage $\delta N/N_m$ of added and actively translocated phospholipid phosphatidylserine (PS). The increase in the number of phospholipids added to the plasma membrane outer layer and specifically translocated to the inner layer induces an increase in the endocytic internalization rate, only after the important gap observed between 1.5% and 2.5% of added phospholipids. (*B*) The endocytic internalization rate as a function of the number of phospholipids added and translocated, at $\phi = 0.2$. The initial slope of the endocytic internalization dynamics under hypoosmotic constraints, α, normalized to its control experiment initial slope α_0, is plotted as a function of the percentage of added PS. A sudden recovery of endocytosis is observed at $\delta N/N_m = 2\%$ and is followed by a linear regime of endocytosis dynamics increase (*black points*). No endocytosis rescue was observed after the addition of 3% lyso-PS, a phospholipid homologous to C6-PS, that is not actively translocated by the flippase activity (*white point*). Each point represents two experiments, except at the transition, where one point is one experiment.

of $R_{cm}/R_{om} = 1.05$. Note that the cell radius R_{cm} remained constant during the endocytic measurements. This ensured that ionic pumps have not completely restored the isotonicity between the cells and the external medium. The elastic surface tension σ_c associated with the membrane dilation α_c at transition can be written as $\sigma_c = 2K\alpha_c = 2K(1 - (R_{0m}/R_{cm})^2) = 0.042\ N \cdot m^{-1}$, where $2K = 0.45\ N \cdot m^{-1}$ (Bloom et al., 1991) is the characteristic elastic modulus of dilation of the plasma membrane for living cells. We thus find, at transition, the following critical pressure asymmetry: $\Delta p_c = 8.4 \times 10^3$ Pa.

The elastic constant of bending for a monolayer of the plasma membrane is $k_c = 10^{-19}$ J (Bloom et al., 1991). The critical pressure asymmetry, $\Delta p_c = (2/3^2)\ (h\Delta\sigma)^3/(16k_c)^2$, deduced from theory, is correlated with the experimental value, $\Delta p_c = 8.4 \times 10^3$ Pa, for $\Delta\sigma = 9 \times 10^{-3}\ N \cdot m^{-1}$,

namely, for a vesiculation radius of $R_1^0 = 16k_c/h\Delta\sigma = 35$ nm. This radius is the endocytic vesicle diameter of 70 nm observed in vivo. Therefore, the observed transition quantitatively correlates with the phase transition predicted by the model, which takes account of the asymmetrical surface tension, given the vesiculation radius of 35 nm observed in vivo.

For a membrane tension asymmetry mechanically induced by the existence of a phospholipid number asymmetry $\Delta N/N_m$ maintained by the pumping activity, we saw that $\Delta\sigma$ can be written as $\Delta\sigma = K(\Delta N/N_m)$. Using $K = 0.225$ N $\cdot$ m^{-1}, $\Delta\sigma = 9 \times 10^{-3}$ N $\cdot$ m^{-1} predicts the existence of a phospholipid number asymmetry at steady state of $\Delta N_0/N_m = 4\%$. This value is physiologically relevant, because 25% of the plasma membrane phospholipids are specifically translocable by the pumping activity (Alberts et al., 1994).

Reverse transition of endocytic vesiculation recovery

From the inversion of the critical pressure asymmetry expression, we predict a recovery transition of endocytic vesiculation at high dilution, at a critical increase of the phospholipid number asymmetry of $\delta N_c/N_m = \Delta N_0/N_m ((\Delta p/\Delta p_c)^{1/3} - 1)$, where Δp is the pressure asymmetry for the dilution ϕ. At $\phi = 0.2$, we measured a mean cell radius R_m of $R_m/R_{0m} = 1.4$, which leads from Laplace's and Hooke's laws to $\Delta p/\Delta p_c = 3.95$. As done previously, we verified that the cell radius R_m remained constant during the experiment. This predicts $\delta N_c/N_m = 2.3 \times 10^{-2}$. This value correlates perfectly with the experimental critical value of $2 \pm 0.5\%$ of added and translocated phospholipids at transition, observed in vivo. This agreement strongly suggests the existence of a membrane tension asymmetry due to the mechanical properties of the plasma membrane, generated and maintained by the phospholipid translocation activity, as a driving force of bulk-flow endocytic vesiculation, in vivo.

DISCUSSION AND PERSPECTIVES

First, the present biological experiments correlate with the proposal of the existence of a membrane tension asymmetry, due to a phospholipid number asymmetry generated by the phospholipid translocation activity, considered to be a driving force of endocytic vesiculation (Farge, 1995; Farge et al., 1999). Effectively, the transition of endocytic vesiculation inhibition under external hypoosmotic pressure, predicted by the model taking account of the membrane tension asymmetry, is experimentally observed in vivo, in quantitative agreement with predictions. The reverse transition of endocytic recovery at low external hypoosmotic pressure ($\Delta p > \Delta p_c$), after the increase in the phospholipid number asymmetry, is also observed, in quantitative agreement with predictions.

Importantly, the addition of lyso-PS, which is not translocated by the flippase activity, did not lead to the recovery transition. Moreover, because it is not recognized by the flippase activity, the lyso-PS is the most specific inhibitor of exogenous PS translocation. As a consequence, adding lyso-PS without osmotic treatment should decrease the membrane tension asymmetry instead of increasing it and consequently inhibit endocytosis. This was effectively observed, as already reported (Farge et al., 1999).

Note that the endocytic vesiculation inhibition under hypoosmotic constraints could have been interpreted in a simpler manner, involving the mean surface tension σ of the plasma membrane due to pressure constraints as the vesiculation inhibition force. In this case, we would have followed a single membrane "liposome-like" model, wherein the plasma membrane could not have been considered to be in contact with a dynamic reservoir of recycling vesicles. This assumption would have necessarily introduced σ into the budding driving force energy Φ_1, following $\Phi_1 = (\sigma - (h/2R)\Delta\sigma)A_0$. As a consequence, the bud energy of Fig. 2 *A* would have been translated by σA_0, so that we would have found $(\partial\phi/\partial A_0)|_{R=R_1} \geq 0$ for $\sigma \geq (2k_c/R_1^2) = 8 \times 10^{-5}$ N $\cdot$ m^{-1}. This would have predicted a transition of budding inhibition at $\Delta p = 2\sigma/R_m = 16$ Pa. This value is four orders of magnitude smaller than the experimental evaluation of the critical pressure asymmetry at transition. This theoretically favors the pressure asymmetry Δp as the direct budding inhibition force.

On the other hand, if we have considered the case where the transition is driven by the mean surface tension, then the addition of C6-PS molecules may have relaxed the membrane tension at high dilution, leading also to the observed endocytosis transition recovery. However, the addition of another phospholipid that is not translocated, the lyso-PS, would have led to such an endocytosis recovery as well. This effect was not observed. This result experimentally favors a role of the pressure asymmetry as the direct budding inhibition force, instead of the plasma membrane mean tension.

Another alternative explanation of the endocytic dynamics recovery transition could be that the addition of C6-PS increases the membrane permeability. In that case, the cells could have relaxed the osmotic pressure asymmetry constraint due to the C6-PS treatment, leading to endocytosis recovery. However, as a detergent-like molecule, the lyso-PS should have permeabilized the cell even more than the C6-PS could. In that case, we should have observed the endocytosis recovery after the addition of lyso-PS. This effect was not observed. On the other hand, we observed no transient internalization of the blue trypan during the hypoosmotic shock in the presence of C6-PS and no decrease in the cell radius after the hypotonic shock in the presence

of C6-PS. Both observations further rule out the alternative hypothesis of a membrane permeabilization.

However, we certainly cannot exclude an indirect biochemical process that could inhibit endocytosis under hypoosmotic constraints, involving, for instance, the opening of calcium channels (Morales-Mulia et al., 1998). But it seems unlikely that the C6-PS would specifically inhibit such indirect biochemical osmotic effects. At the moment, the model taking account of the elastical properties of the plasma membrane probably remains the most suitable hypothesis and, moreover, quantitatively predicts both observed transitions.

Finally, the present results do not exclude alternative mechanisms for the generation of endocytic vesicles. First, endosomal pH asymmetries could also contribute to the formation of cytosolic vesicles. Given that these membranes contain the phospholipid phosphatidic acid (PA) (Jones et al., 1997), which should be translocated from the acid luminar monolayer to the basic cytosolic monolayer (Redelmeir et al., 1990), the pH asymmetry could initiate the formation of cytosolic vesicles through its effect on the asymmetrical number of phospholipids. This hypothesis is supported by the observation that liposomes containing acidic phospholipids spontaneously give rise to small vesicles, in response to a transmembrane pH gradient (Farge and Devaux, 1992). In this case, ionic proton pumps would play the role of an indirect phospholipid pump, leading to the pH asymmetry, which is the driving force for the PA translocation. Interestingly, the aminophospholipid translocase has been suggested to have diverged from the family of ion translocating ATPases (Tang et al., 1996). From an evolutionary point of view, the PA translocation in response to pH asymmetries might thus have been one of the first cell budding driving forces. It might also have been one of the simplest ways of generating buds in prebiotic cells.

Second, a membrane tension asymmetry could also be generated by an asymmetry of electrical charge density between the two sides of the plasma membrane, controlled, for instance, by ionic pumps. Indeed, because PS is charged, one cannot exclude a purely electrostatic origin for $\Delta\sigma$ in the present experiments. On the other hand, the interaction of clathrin or claveole molecules solely with the inner phospholipid monolayer may generate a membrane tension asymmetry as well, leading to the plasma membrane bending (Matsuoka et al., 1998). Alternatively, either the cell surface-to-volume ratio (Käs and Sackmann, 1991) or the local phase segregation of lipids (Lipowsky, 1993; Jülicher and Lipowsky, 1996; Döbereiner et al., 1993) might also lead to the vesiculation of the plasma membrane in vivo.

Here we theoretically (as well as experimentally in vivo) propose the following hypothesis for endocytic vesiculation: from the coupling between the biochemical transmembrane phospholipid pumping activity and the physical properties of the plasma membranes, considered as soft matter, emerges a physiological process of vesiculation, the first step of the cell endocytosis process.

Importantly, the vesiculation phase transition we found in response to the generation of an osmotic pressure asymmetry, could be used by the cell to mechanically control "on-and-off" cell genetic responses in the presence of cytokines. Effectively, cytokines are signaling proteins secreted by surrounding cells or by the cell itself. They bind to their specific plasma membrane receptors and trigger specific cell genetic transcriptions. These cell genetic transcriptions are thought to strongly depend on the internalization of the cytokine-receptor complex (see the Introduction). An "on-and-off" switch of the cytokine endocytic internalization, controlled by a modulation of the pressure asymmetry around the transition, could very precisely switch the cell genetic transcription response to the cytokine on or off. Our evaluation of the critical pressure asymmetry at transition, $\Delta p_c \approx 8 \times 10^3$ Pa, represents only 0.1% of the $p_0 = 7.5 \times 10^6$ Pa isotonic osmotic pressure. Such volumic pressure asymmetry could probably be generated actively by cell plasma membrane ionic pumps or other processes known to regulate the internal osmotic pressure of the cell (Sweadner and Goldin, 1980). It could also be actively generated by an increase in the hydrostatic pressure in internal medium, due to cytoskeleton contractions (Raucher and Sheetz, 1999). In that case, from the coupling between biochemical ionic pumping activities or active cytoskeleton constraints and the elastic properties of the plasma membranes physically considered as soft matter may emerge a physiological process of on-and-off control of endocytic vesiculation, switching with an accurate precision between cell genetic transcription responses to cytokines. Note that most of the cytokine receptors internalize cells through the clathrin-dependent pathway. Here we theoretically analyzed and measured bulk-flow endocytosis. As a consequence, such a mechanism for genetic transcription control presumes the same behavior for clathrin-dependent endocytosis.

Finally, we saw that a dilution of a factor of $\sim 1/2$ of the external medium could lead to the value of the critical pressure asymmetry. This means that a decrease in the external environmental osmotic pressure of a factor of 1/2 is potentially able to switch on or off a cell genetic transcription response dependent on cytokines. In this case, the external environmental osmotic pressure could control the genetic responses to external signaling molecule cytokines. The transition could thus also be considered to be one process of epigenetic gene expression regulation, under physicochemical external osmotic constraints, in the presence of an external soluble signaling protein.

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