Transbilayer phosphatidylethanolamine movements in the yeast plasma membrane

Evidence for a protein-mediated, energy-dependent mechanism

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Aminophospholipid movements in the plasma membrane of higher eukaryotic cells seem to be regulated by an ATP-dependent, protein-mediated process. To examine whether similar mechanisms exist in yeast cells, we have analysed phosphatidylethanolamine (PtdEtn) distributions in Saccharomyces cerevisiae (A184D) cells under a variety of conditions, with trinitrobenzenesulphonic acid and fluorescamine as the external membrane probes. The levels of external PtdEtn in the intact cells were reduced to about 50% by pretreatment of the cells with inhibitors of mitochondrial ATP synthesis, ATPase inhibitors or protein-sulphydryl-group-modifying reagents, or by depletion of the cells of ATP by metabolic starvation. The levels of external PtdEtn could be restored to normal by repletion of the energy-depleted cells with ATP. Furthermore, treatment of the energy-depleted cells with sulphydryl-modifying reagents did not cause further reduction in the external PtdEtn levels but decreased the accessibility of PtdEtn to fluorescamine after restoration of the cellular ATP levels to normal in these cells. These results demonstrate an involvement of an ATP-dependent, protein-mediated process(es) in the regulation of the PtdEtn distribution across the plasma-membrane bilayer of yeast cells. The results are discussed with regard to possible models that can generate and maintain the transbilayer phospholipid asymmetry in the yeast plasma membrane.

Keywords: aminophospholipid asymmetry; transbilayer phospholipid movement; aminophospholipid translocase; yeast plasma membrane.

Transbilayer phospholipid movements in biological membranes give rise to large concentration gradients for the various lipid components across the membrane bilayer. The apparent stability of such gradients suggest that these must have some functional significance. Several biological phenomenon, such as membrane biogenesis, membrane fusion, endocytosis, exocytosis, regulation of membrane protein activity, modulation of surface potential, procoagulant activity of blood platelets, and the clearance of the aged and diseased cells from the circulation, have been suggested to involve transbilayer redistribution of membrane phospholipids [1–9]. Earlier studies proposed that the asymmetric distribution of plasma membrane phospholipids in higher eukaryotic cells is generated and maintained by an ATP-dependent out-to-in aminophospholipid pump, possibly in association with selective interactions of the inner-phospholipids with the underlying cytoskeletal proteins [1, 3, 4, 10–12].

To further analyse the mechanisms of generation and maintenance of membrane-phospholipid asymmetry in the eukaryotic cells, it seems essential to have a model system in which well-defined structural changes can be introduced in a given cytoskeleton of the target gene. Therefore, Saccharomyces cerevisiae appears to be an ideal cell to study, since it has a well-defined cytoskeletal apparatus [13], has a plasma-membrane-phospholipid asymmetry that is typical of the higher eukaryotic cells [14], and is genetically well characterized. However, it is not known whether S. cerevisiae, similarly to higher eukaryotic cells, possess an aminophospholipid pump that can translocate phosphatidylethanolamine (PtdEtn) and phosphatidylserine (PtdSer) across the plasma-membrane bilayer. Here, we show that at least the PtdEtn movements across the yeast plasma membrane bilayer are regulated by ATP-dependent, protein-mediated process(es).

MATERIALS AND METHODS

Materials. Yeast extract and mycological peptone (peptoneM) were from Hi Media Laboratories. Yeast Nitrogen Base without amino acid supplements was bought from Difco Laboratories. Adenine sulfate, uracil, trinitrobenzenesulphonic acid, fluorescamine, sodium azide, sodium orthovanadate, di-thiothreitol, N-ethylmaleimide, miconazole nitrate, diethylstilbestrol, chloramphenicol, cycloheximide, 9-aminoacridine, digitonin, Triton X-100, ATP estimation kit (366-UV), BSA, and L- amino acids were purchased from Sigma Chemical Company. Coomassic Brilliant Blue G-250 was obtained from Bio-Rad Laboratories. Precast aluminium-backed silica-gel 60 F254 TLC plates were obtained from E. Merck. L-[55S]-methionine (>1000 Ci/mmol) was from Bhabha Atomic Research Centre. All other reagents were of the highest purity available.

The yeast strain A184D was a generous gift from Dr M. Bard, Department of Biology, Indiana University, USA.
Growth media and growth conditions. Yeast cell stocks were maintained on 1% yeast extract, 2% peptone M, 2% d-glucose, pH 5.4 (medium A)/2% agar slants at 4°C. Yeast cultures were grown in medium A at 30°C in an Infors orbital-shaker incubator. Consistent aeration was provided by constant swirling of the conical flasks at 200 rpm, the volume of medium being 20% that of the flask for all experiments. All cultures were grown to late log phase (3.6 mg dry cell mass/ml media) to ensure a homogeneous population of cells.

Trinitrobenzenesulfonic acid labeling. Yeast cells were harvested by centrifugation at 1000×g for 3 min at 25°C and washed twice with 5 vol. 100 mM potassium phosphate, 5 mM EDTA, pH 7.5 (buffer A). Washed cells were suspended in 100 mM potassium phosphate, 600 mM KCl, pH 8.2 (buffer B), to 2×10⁸ cells/ml. The suspension was transferred to a 50-ml glass conical flask and cooled to 10°C by gentle swirling for 10 min in a water bath at 10°C. An equal volume of prechilled freshly prepared trinitrobenzenesulfonic acid solution (20 mM in buffer B) was added, and the cells were incubated at 10°C with constant swirling to keep them in suspension. At different times, 10 ml suspension was mixed with 10 ml 200 mM potassium acetate, 600 mM KCl, pH 4.5 (buffer C), and centrifuged at 1000×g for 5 min at 2°C. The cell pellet was washed with buffer C till the supernatant was free of color.

Fluorescamine labeling. To washed cells (2.2×10⁸ cells/ml) in 3 ml of buffer B, fluorescamine (about 0.8 M) in dry dimethylsulfoxide (Me₂SO) was added dropwise under constant swirling. After 30 s, an equal volume of 1 M ammonia in 600 mM acetic acid, pH 4.5 (buffer C), and centrifuged at 1000×g for 5 min at 2°C. The cell pellet was washed with buffer C till the supernatant was free of color.

Sodium azide treatment. All operations were performed under sterile conditions. Late-log-phase cells were harvested, washed twice with 5 vol. H₂O, and suspended to 1×10⁹ cells/ml in synthetic complete medium [15] containing 2% D-sorbitol (medium B) and different concentrations of sodium azide. The suspensions were incubated at 37°C for 2 h with constant swirling at 200 rpm in an Infors orbital-shaker incubator, after which they were processed for fluorescamine labeling and ATP estimation. A portion of the cell suspension was removed prior to incubation and processed for fluorescamine labeling and ATP estimation. The results obtained were taken as the control values.

Sodium orthovanadate treatment. To the washed cells (2×10⁹ cells/ml) in 3 ml of buffer B, fluorescamine (about 0.8 M) in dry dimethylsulfoxide (Me₂SO) was added dropwise under constant swirling. After 30 s, an equal volume of 1 M ammonia in 600 mM potassium acetate, 600 mM KCl, pH 4.5 (buffer C), and centrifuged at 1000×g for 5 min at 2°C. The cell pellet was washed with buffer C till the supernatant was free of color.

Diethylstilbestrol/miconazole nitrate treatment. To the washed cells (1×10⁹ cells/ml) suspended in medium C containing 2% d-glucose (medium C), buffered with Mes, pH 6.0, sodium orthovanadate was added. The mixture was incubated at 37°C for 2 h with constant swirling at 200 rpm in an Infors orbital-shaker incubator, after which they were processed for fluorescamine labeling and ATP estimation. A portion of the cell suspension was removed prior to incubation and processed for fluorescamine labeling and ATP estimation, and the results obtained were taken as the control values.

Sodium orthovanadate treatment. To the washed cells (2×10⁹ cells/ml) in 3 ml of buffer B, fluorescamine (about 0.8 M) in dry dimethylsulfoxide (Me₂SO) was added dropwise under constant swirling. After 30 s, an equal volume of 1 M ammonia in 600 mM potassium acetate, 600 mM KCl, pH 4.5 (buffer C), and centrifuged at 1000×g for 5 min at 2°C. The cell pellet was washed with buffer C till the supernatant was free of color.

N-Ethylmaleimide treatment. Yeast cells were washed twice with 5 vol. 50 mM potassium phosphate, pH 7.6 (buffer D) and suspended in medium C containing 50 mM potassium phosphate, pH 7.5 (1°C) to 1.1×10⁹ cells/ml. The temperature of the suspension was reduced to about 1°C by incubation in an ice bath for 10 min with gentle swirling. N-Ethylmaleimide (2 mM) in the same buffered medium was added to the cell flasks. The suspensions were incubated with gentle swirling for the required times, after which the cells were harvested by centrifugation at 1000×g (2°C, 5 min), washed once with buffer A containing 2 mM dichothioretic acid and twice with buffer A, and immediately processed for fluorescamine labeling.

Metabolic depletion of ATP. All operations were performed under sterile conditions. Late-log-phase cells were harvested, washed twice with 5 vol. distilled water and suspended in medium B to 1×10⁹ cells/ml. The suspension was incubated at 37°C with constant swirling at 200 rpm, for the different time intervals, after which a small volume of the sample was taken out and processed for fluorescamine labeling and ATP estimation. A small amount of the cell suspension was harvested prior to incubation and processed for fluorescamine labeling and ATP estimation. The results thus obtained were taken as the control values.

ATP repletion. To 10 ml of the suspension of ATP-depleted cells (1×10⁹ cells/ml), cycloheximide and chloramphenicol were added to final concentrations of 0.2 mg/ml each. The cells were incubated at 25°C for 15 min with gentle swirling, after which a sterile solution of D-glucose was added to the cell suspension to give a final concentration of 2% (mass/vol.). The cells were incubated at 37°C (200 rpm) and processed for fluorescamine and ATP estimation.

Measurement of protein synthesis. The extent of protein synthesis in intact yeast cells was determined as the amount of trichloroacetic-acid-precipitable radioactive contamination after incubation in the media containing L-[³⁵S]methionine. Intact yeast cells were incubated with L-[³⁵S]methionine for 3 h. The cells were harvested by centrifugation at 1000×g for 5 min at 25°C. The cell pellet was washed twice with 5 vol. buffer A and suspended in 100 mM potassium phosphate, 600 mM KCl, 15 mM 2-mercaptoethanol, pH 7.5 (buffer E), at 10 mg dry cell mass/ml. The suspension was incubated at 30°C for 30 min. A freshly prepared suspension of Zymolyase-100T in buffer E was added at a ratio of 5 mg/g dry cell mass. The incubation was continued at 30°C for 60 min, after which the proteins in the suspension were precipitated by the addition of trichloroacetic acid to 30%. After incubation on ice for 15 min, the suspension was centrifuged at 13000 rpm for 10 min at 4°C in a microcentrifuge, and the pellet washed twice with diethyl ether to remove the excess trichloroacetic acid. Residual ether was removed from the precipitate by slight warming and solubilized in 100 μl 5 M sodium hydroxide. A small quantity of the solubilized pellet was used to measure the ³⁵S radioactivity. Radioactivity was normalized to protein concentration, and the extent of protein synthesis was expressed as the fraction of the initial counts that were incorporated as L-[³⁵S]methionine labeled protein.

Measurement of plasma-membrane surface potential. The surface potential at the plasma-membrane outer leaflet was determined by a modification of the method used by Cerbón and Calderón [16]. Late-log-phase yeast cells were harvested, washed three times with 20 mM Tris/citrate, pH 6.0, and suspended to 12, 6 or 2 mg/ml dry cell mass in 20 mM Tris/citrate, pH 6.0, 2 μM 9-aminoacridine (excitation and emission wavelengths, 400 nm and 454 nm, respectively). Diethylstilbestrol or miconazole nitrate was added to 200 μM. The decrease in fluorescence intensity at 454 nm was recorded for up to 20 min and the fluorescence was dequenched by the addition of magnesium chloride to 100 mM. The dequenched fluorescence intensity was taken as the maximum fluorescence and the fluorescence intensities were expressed as percentages of this value.

Protein estimation. Total protein in yeast spheroplasts and intact cells was estimated essentially according to the methods of Bradford [17] and Cordeiro et al. [18], respectively.
Estimation of ATP levels. A cell suspension (equivalent to 1.7×10^6 cells) was centrifuged in a microcentrifuge and the pellet washed twice with water. The washed pellet was suspended in 120 μl 0.5% digitonin in water, and vortexed vigorously for 15 min at 25°C in an eppendorf vibrato-mixer. 20 μl permeabilised cells were kept aside for protein estimation, and the remaining suspension made to 10% trichloroacetic acid by the addition of 20 μl concentrated trichloroacetic acid. The trichloroacetic-acid-treated sample was vortexed, incubated on ice for 10 min and centrifuged. 10 μl supernatant were mixed with 20 μl 500 mM NaOH. The amount of ATP in the extract was determined by means of a Sigma ATP-estimation kit (Procedure no. 366-UV).

Lipid extraction, TLC and phosphate estimation. Lipids were extracted essentially by the Folch procedure [9]. TLC was performed on precoated silica-gel G-60 plates. The solvent systems used were as follows: first dimension, chloroform/methanol/25% ammonia (65:25:6); second dimension, chloroform/methanol/acetone/acetic acid/water (40:8:16:9:4). Plates were stained with iodine vapors and the spots corresponding to the various phospholipids and derivatised phospholipids cut out carefully. Lipid phosphate was estimated according to the method of Bartlett [20].

RESULTS

Evaluation of a suitable reagent for labeling of plasma-membrane PtdEtn in intact yeast cells. Of the various aminolipid-labeling reagents, trinitrobenzenesulfonic acid and fluorescamine are particularly useful. Trinitrobenzenesulfonic acid has been used widely to determine the aminophospholipid distribution in higher eukaryotic cells, viruses, bacteria, yeast and intracellular organelles [21]. But fluorescamine seems to be a better labeling reagent than trinitrobenzenesulfonic acid, because its reactions proceed very rapidly [22]. This feature, coupled with its poor stability in water, makes fluorescamine extremely useful for capturing membrane events with minimal alterations in the lipid organization. This reagent has been used to label red cells [23–25] and microsomal membranes [26]. Figs 1 and 2 illustrate the labeling of yeast-cell PtdEtn with trinitrobenzenesulfonic acid and fluorescamine, respectively. Both reagents labeled 10–14% PtdEtn in intact yeast cells, whereas under identical conditions about 70% PtdEtn was modified in leaky cells. Prolonged incubation (6–8 h) with trinitrobenzenesulfonic acid was required to achieve the saturable levels of labeling compared with 30 s for fluorescamine.

Phospholipid composition of the yeast cells. Table 1 shows the relative proportions of the four major phospholipids in A184D cells. Since the phospholipid composition of yeast cells is known to be influenced by several environmental factors, such as media composition, pH, temperature, aeration and the growth stage of the cells [27], which could lead to alterations in the membrane-phospholipid organization, care was taken to ensure that the experimental procedures adopted did not bring about changes in the lipid composition.

Table 1. Proportions of the major phospholipids of A184D cells. Values are means ± S. D. of 12 observations.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Proportion</th>
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<tr>
<td>PtdSer</td>
<td>11.83 ± 1.98</td>
</tr>
<tr>
<td>PtdEtn</td>
<td>23.23 ± 1.03</td>
</tr>
<tr>
<td>PtdIns</td>
<td>16.69 ± 1.12</td>
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<td>PtdCho</td>
<td>50.40 ± 2.35</td>
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Effects of sodium azide and ATPase inhibitors on the external plasma-membrane PtdEtn levels. Effect of sodium azide. Sodium azide is an inhibitor of mitochondrial ATP synthesis, and has been widely used to effect ATP depletion. Yeast cells were incubated in medium B containing 0–20 mM sodium azide as described in Materials and Methods. Fig. 3A shows the kinetics of ATP depletion in control cells and in cells treated with sodium azide. A rapid concentration-dependent reduction of cellular ATP was observed, with the ATP levels reaching a minimum at about 10% of the control values after 2 h. Fluorescamine labeling of the azide-treated cells resulted in a decrease of external PtdEtn levels compared with those of control cells (Fig. 3B). Since this decreased labeling could result from a lower reactivity of the phospholipid with fluorescamine, the azide-treated cells were labeled with fluorescamine after rendering them leaky with 0.5% Triton X-100. Under these conditions,
Fig. 3. Sodium azide treatment decreases the accessibility of the external PtdEtn to fluorescamine. (A) Kinetics of ATP depletion in sodium-azide-treated A184D cells. Cells suspended in medium C (●); medium B (△); medium B +5 mM azide (▽); medium B +10 mM azide (■) and medium B +20 mM azide(□) (B) Effect of sodium azide on the accessibility of PtdEtn to fluorescamine labeling in intact yeast cells. Cells were treated with sodium azide and labeled with 60 μmol fluorescamine for 30 s, as described in Materials and Methods. More than 35% PtdSer and more than 70% PtdEtn were derivatized in sodium-azide-treated cells that were permeabilized with 0.5% Triton X-100 under identical conditions.

Fig. 4. ATPase inhibitors decrease the accessibility of external PtdEtn to fluorescamine. Effect of orthovanadate (A), diethylstilbestrol (B) and miconazole nitrate (C) on the accessibility of PtdEtn to fluorescamine labeling in intact yeast cells. Cells were treated with orthovanadate, diethylstilbestrol or miconazole nitrate and labeled with 60 μmol fluorescamine for 30 s, as described in Materials and Methods. More than 35% PtdSer and more that 70% PtdEtn were derivatized in the inhibitor-treated cells that were permeabilized with 0.5% Triton-X-100 under identical conditions.

extensive labeling of PtdEtn and PtdSer was observed, which eliminated the possibility of decreased reactivity of these aminophospholipids towards fluorescamine in the azide-treated cells. From these results, we infer that the observed decrease in PtdEtn levels could be due to a decrease in the amounts of external PtdEtn in the ATP-depleted cells.

Effect of sodium orthovanadate. Sodium orthovanadate is an inhibitor of most of the nucleotide-dependent processes and is thought to exert its effect by mimicking the phosphate group at the nucleotide-binding site [28]. It has been used to inhibit the activity of the aminophospholipid translocase in partially purified membrane fractions [29] and intact cells [6, 30], and is hence a good choice for the present studies. Yeast cells were incubated with different concentrations of sodium orthovanadate as described in Materials and Methods. Up to 5 mM vanadate did not alter the amount of external PtdEtn (Fig. 4 A), presumably due to poor permeability of the anion across the yeast plasma membrane (as observed in higher eukaryotic cells [6, 30]) and the presence of the overlying complex cell-wall structure. However, above 5 mM, a gradual vanadate-concentration-dependent decrease in PtdEtn levels was observed. This decrease was not due to the shielding of the fluorescamine-reactive sites on PtdEtn by vanadate, as detergent-permeabilized cells exhibited extensive labeling of the aminophospholipids, even at 20 mM

Fig. 5. Gross transbilayer lipid redistribution is accompanied by an alteration in 9-aminoacridine binding. The effect of miconazole nitrate and diethylstilbestrol on the lipid redistribution, as a result of gross membrane destabilization, was determined by measurement of the change in surface potential of the cells, as described in Materials and Methods. Cells/miconazole nitrate (mg dry cell mass/mmol), 120:2 (□); 60:2 (●); 20:2 (△); cells/diethylstilbestrol (mg dry cell mass/mmol), 120:2 (▽); 20:2 (○).
Fig. 6. Protein-modification reagents decrease the accessibility of external PtdEtn to fluoroscamine. Effect of the duration of the N-ethylmaleimide labeling (A) and N-ethylmaleimide concentration (B) on the accessibility of PtdEtn to fluoroscamine. Cells were treated with N-ethylmaleimide and labeled with 60 μmol fluoroscamine for 30 s, as described in Materials and Methods. More than 35% PtdSer and more than 70% PtdEtn were derivatized in N-ethylmaleimide-treated cells that were permeabilized with 0.5% Triton X-100 under identical conditions.

Fig. 7. Reversible depletion of metabolic stores brings about a reversible reduction in the external PtdEtn levels. (a) Effect of metabolic depletion/repletion on the accessibility of PtdEtn to fluoroscamine in intact yeast cells. Cells were incubated in medium B for 0 (A), 10 (B) or 12 h (C), and labeled with 60 pmol fluoroscamine for 30 s at 10°C. Energy-depleted cells (B) were repleted for energy by incubation with D-glucose for 90 min (D) or 180 min (E) and labeled with 60 μmol fluoroscamine for 30 s. More than 35% PtdSer and more than 70% PtdEtn were derivatized in ATP-depleted cells that were permeabilized with 0.5% Triton X-100. The inset shows the kinetics of ATP depletion in A184D cells. Cells incubated in medium C (control cells) (D) and medium B (B). (b) Effect of protein-synthesis inhibitors on the reversal of PtdEtn accessibility to fluoroscamine in energy-depleted/repleted yeast cells. Cells (A) were depleted of ATP by incubation at 37°C in medium B for 10 h (B). The ATP-depleted cells were repleted for ATP by the addition of D-glucose in the absence (C) or presence (D) of chloramphenicol and cycloheximide (0.2 mg/ml each), and labeled with 60 μmol fluoroscamine for 30 s. More than 35% PtdSer and more than 70% PtdEtn were labeled with fluoroscamine in cells that were permeabilized with 0.5% Triton-X-100 under identical conditions. The inset shows the incorporation of L-[35S]methionine in ATP-depleted cells in the absence and presence of protein-synthesis inhibitors. (c) Effect of ATP-depletion and N-ethylmaleimide treatment on the accessibility of PtdEtn to fluoroscamine in yeast cells. Cells (A) were depleted of ATP (B), treated with N-ethylmaleimide (C), and labeled with 60 μmol fluoroscamine for 30 s, as described in Materials and Methods. To a small portion of the ATP-depleted cells, D-glucose was added, and the mixture incubated at 37°C for 3 h, after which the cells were harvested and labeled with 60 μmol fluoroscamine for 30 s (D). A small portion of ATP-repleted cells was incubated with N-ethylmaleimide, and the N-ethylmaleimide-treated cells were labeled with 60 μmol fluoroscamine for 30 s (E). More than 35% PtdSer and more than 70% PtdEtn were labeled by fluoroscamine in the cells that were permeabilized with 0.5% Triton X-100 under similar conditions.

vanadate. These results indicate that the transbilayer PtdEtn distribution in the yeast plasma membrane is perhaps modulated by an ATPase activity. To further establish the involvement of ATPase activity in transbilayer PtdEtn movements, two well-known ATPase inhibitors, namely diethylstilbestrol and miconazole nitrate, were used.

Effect of diethylstilbestrol. Diethylstilbestrol is widely used as an inhibitor of the plasma-membrane proton ATPase in yeast and fungi [31]. However, the exact mechanism of its inhibitory action is not known. Yeast cells were incubated with different concentrations of diethylstilbestrol as described in Materials and Methods.

A concentration-dependent decrease in PtdEtn labeling was observed compared with that of control cells (Fig. 4B). Incubation periods were restricted to 10 min, because prolonged incubations (up to 30 min) lead to the formation of appreciable amounts of lysolipids.

Effect of miconazole nitrate. Miconazole nitrate is used as an inhibitor of the plasma-membrane H+-ATPase in yeast. It is known to exert its effect by blocking the phosphorylation/phosphorylation reactions [32]. Yeast cells were treated with varying concentrations of miconazole nitrate, as described in Materials and Methods. A concentration-dependent decrease in PtdEtn labeling was observed, which reached a maximum at about 200 μM miconazole nitrate (Fig. 4C). Incubation of the yeast cells with this inhibitor resulted in formation of lysolipids. Hence, the incubation periods were restricted to 10 min at 30°C to minimize lysolipid formation.
Miconazole nitrate has been reported to alter the membrane integrity and to cause alterations in the lipid organization [33–35]. It is therefore essential to determine whether the observed effects of this inhibitor on PtdEtn distribution were due to inhibition of ATPase activity or due to non-specific transbilayer phospholipid redistribution. Since the negative surface potential of yeast cells has been attributed to the presence of negatively charged phospholipids on the outer surface of the plasma membrane [14, 16, 36, 37], gross transbilayer redistribution of the plasma-membrane phospholipids may be expected to result in changes in the surface potential, which may be measured by means of 9-aminoacridine [16]. At the inhibitor/cell ratios used in the study (2 μmol/120 mg dry cell mass), no gross alterations in 9-aminoacridine binding occurred (Fig. 5), which confirmed that the observed effects on the PtdEtn labeling were due to an inhibition of the ATPase activity, rather than to non-specific lipid scrambling.

**Effect of N-ethylmaleimide treatment on the external plasma-membrane PtdEtn levels.** Since N-ethylmaleimide reacts with free thiol groups present in proteins, any alteration in membrane-lipid organization after N-ethylmaleimide treatment may be attributed to inhibition of a protein-mediated process(es). This approach has been used widely to determine the mechanism of transbilayer lipid asymmetry in eukaryotic cells [38, 39]. Covalent modification of yeast cells with N-ethylmaleimide affected about a 40% reduction in the external PtdEtn, compared with the control levels (Fig. 6).

**Effects of ATP depletion/repletion on the external plasma-membrane PtdEtn levels.** Effect of ATP depletion. Yeast cells were depleted of ATP by incubation in media lacking glucose, as described in Materials and Methods. Fig. 7a shows the kinetics of ATP depletion. ATP levels fell to about 45% of the initial values within 2 h. After 2 h, the decrease in cell ATP was rather slow, with about 16% remaining even after 16 h of the incubation. Analysis of the phospholipid composition of cells incubated in the absence of D-glucose showed that incubation beyond 13 h resulted in marked alterations in the phospholipid compositions (data not shown). However, it was possible to achieve about 80% depletion of ATP in 12 h and to keep the phospholipid composition identical to that at the start of the incubation. Therefore, we determined the transbilayer PtdEtn distribution after only 10 h and 12 h of metabolic starvation.

Metabolic starvation of yeast cells for 10 h effected a 50% reduction in the amount of external PtnEtn (Fig. 7a). Increasing the incubation period to 12 h did not further reduce the extent of PtdEtn labeling. To further establish whether this reduced level of external PtdEtn was due to the reduced ATP levels, it was considered necessary to determine the extent of PtdEtn labeling after repletion of ATP in ATP-depleted cells. Effect of ATP repletion. Metabolically starved cells were repleted with ATP by addition of D-glucose and incubation under the same conditions. The amount of accessible outer-leaflet PtdEtn in the ATP-repleted cells increased from 49% to 73% of the levels in control cells within 90 min of incubation, and to 95% within 180 min (Fig. 7a). To eliminate the possibility that this increase in PtdEtn labeling was due to the synthesis of fresh copies of the effector proteins during ATP repletion, repletion experiments were carried out in the presence of protein-synthesis inhibitors. Despite inhibition (>95%) of protein synthesis, the fraction of the external PtdEtn accessible to fluorescamine in ATP-repleted cells was not affected (Fig. 7b). Furthermore, it was observed that treatment of the ATP-depleted/repleted cells with N-ethylmaleimide resulted in decreased accessibility of the external PtdEtn to the labeling reagents (Fig. 7c). It would, therefore, seem that the N-ethylmaleimide labeling and the ATP depletion have the same site of action.

**DISCUSSION**

The results presented here indicate that fluorescamine can be conveniently employed to determine the transbilayer PtdEtn distributions in plasma membranes of intact yeast cells. That this reagent labels only the external PtdEtn in these cells is strongly suggested by our finding that, under identical conditions, over 70% of the yeast-cell PtdEtn is modified in leaky cells. Examination of the fluorescamine-labeled cells under a light fluorescence microscope (Leitz) revealed that the fluorescence in the labeled intact cells is confined to the surface of these cells, whereas it is highly diffused in the leaky cells (Dixit, B. L., and Gupta, C. M., unpublished observations). Furthermore, yeast cells maintain their cytoplasmic pH at around 6.3, which is much lower than the optimal pH required for the fluorescamine labeling (pH 8). It is known that even when the external pH is as high as 8.2, the cytoplasmic pH in yeast cells does not increase beyond 7.0 [40, 41]. Moreover, this increase in pH occurs only after about 20 min of incubation in alkaline buffers [41], which is well after the time for the fluorescamine labeling (30 s).

This study shows that treatment of yeast cells with mitochondrial-ATP-synthesis inhibitors (e.g., sodium azide) or ATPase inhibitors (e.g., sodium orthovanadate, diethylstilbestrol or miconazole nitrate) leads to a decrease in the external PtdEtn levels. Further, it demonstrates that a similar decrease in PtdEtn levels may be effected by treatment of the cells with the sulphydryl-modifying reagent, N-ethylmaleimide, or by depleting the cells of ATP. The external PtdEtn levels may be restored to normal by repleting the ATP levels in ATP-depleted cells. These results strongly suggest that the transbilayer PtdEtn movements in the plasma membrane of the yeast cells are mediated by an ATP-dependent process. That this process requires the involvement of protein(s) is demonstrated by our observation that N-ethylmaleimide treatment affects the external PtdEtn levels in normal and ATP-repleted cells, but not in ATP-depleted cells. This proposal is supported by the results from our studies on the effect of ATPase inhibitors on the external PtdEtn levels in intact yeast cells.

Several workers have shown that about 60% of the total yeast phospholipid belongs to the plasma membrane [42–45]. Based on this presumption, the present results indicate that about 80% of the plasma-membrane PtdEtn is localized in the inner monolayer. This value is slightly lower than that reported earlier by Cerbon and Calderón for the yeast S. carlsbergensis [36]. These workers further showed that 85% of the phosphatidylinositol (PtdIns) and 90% of the PtdSer are localized in the inner leaflet of the plasma-membrane bilayer [36]. We were, however, unable to detect any external PtdSer in normal or modified yeast cells under our experimental conditions.

The steady-state transbilayer distribution of phospholipids may be considered to be selected from the selective localization of certain phospholipids in the inner or outer leaflet of the plasma-membrane bilayer, which could be maintained by any of the 8 processes shown in Fig. 8a. Routes (1) and (2) are N-ethylmaleimide-insensitive, energy-dependent processes, routes (3) and (4) are N-ethylmaleimide-sensitive, energy-independent processes, routes (5) and (6) exhibit N-ethylmaleimide sensitivity and energy dependence, and routes (7) and (8) involve neither an N-ethylmaleimide-sensitive nor an energy-dependent process, but are regulated by other properties, such as transmembrane poten-
Fig. 8. Models for the mechanism of regulation of the dynamic lipid asymmetry. (a) Possible mechanisms that might maintain the dynamic lipid asymmetry. Energy-dependent and N-ethylmaleimide insensitive (A), energy independent and N-ethylmaleimide sensitive (B), energy dependent and N-ethylmaleimide sensitive (C), and transmembrane potential, transmembrane pH gradient or asymmetrically polarized proteins (D). E, energy; NEM, N-ethylmaleimide. (b) Possible models for maintenance of dynamic lipid asymmetry in the yeast plasma membrane. An energy-dependent, N-ethylmaleimide-sensitive in-to-out flux that is slower than the energy dependent out-to-in flux, which is N-ethylmaleimide-insensitive (i); an energy-dependent, N-ethylmaleimide-sensitive in-to-out flux that is countered by the movement of the lipids from out-to-in under the influence of a potential gradient, pH gradient and/or asymmetrically charged protein (ii); an energy-independent, N-ethylmaleimide-sensitive in-to-out flux and an energy dependent out-to-in flux, which is N-ethylmaleimide insensitive (iii); and an energy-independent N-ethylmaleimide-sensitive in-to-out flux that is countered by the movement of the lipids from out-to-in under the influence of potential gradient, pH gradient and/or asymmetrically charged protein (iv). E. energy; NEM, N-ethylmaleimide.

tial, transmembrane pH gradient, or asymmetric charge distribution in proteins, which might mediate specific or non-specific lipid movements. Since any of these routes might operate, 16 possible combinations of the in-to-out and out-to-in lipid-translocating mechanisms are possible.

Since N-ethylmaleimide treatment results in a decreased accessibility of the outer-leaflet PtdEtn to fluorescamine, it is logical to expect that the in-to-out movements of PtdEtn are sensitive to this covalent modification rather than the out-to-in flux. The possibility that both the processes are N-ethylmaleimide sensitive was eliminated, as this possibility should either lead to symmetric PtdEtn distribution across the yeast plasma membrane bilayer, which would lead to an increased PtdEtn labeling by fluorescamine, or should ‘freeze’ the steady-state distribution, which would lead to no changes in the PtdEtn labeling. The results of N-ethylmaleimide labeling, therefore, reduce the number of possible combinations to four (Fig. 8b), which are further discussed below.

Mechanisms (i) and (iii). The yeast plasma membrane may be considered to have a bidirectional energy-driven lipid-pumping mechanism that maintains a high concentration of PtdEtn in the inner leaflet (80%), compared with 20% in the outer leaflet. The pump responsible for the in-to-out movement is differentiated from its out-to-in counterpart by its N-ethylmaleimide sensitivity and lower efficiency of lipid translocation, i.e., the outward flux is brought about by an N-ethylmaleimide-sensitive low-affinity ATPase (pump 1), compared with the inward translocation, which is perhaps assisted by an N-ethylmaleimide-insensitive high-affinity ATPase (pump 2). Both the pumps function such that a high concentration (~80%) of PtdEtn is maintained in the inner leaflet. Treatment of the cell with N-ethylmaleimide blocks the outward movement of PtdEtn by arrest of pump 1 activity through covalent modification. This blockade should result in a net inward movement of PtdEtn (the outward movements may occur by simple diffusion across the bilayer, which is relatively slow), thereby leading to the decreased PtdEtn labeling. 80% depletion of the ATP levels would reduce the efficiency of both the pumps. However, due to the higher capacity of pump 2, the inward flux takes precedence over the outward movements. These observations cannot, however, be explained if the outward flux is considered to be energy independent [mechanism (ii)]. However, the above hypothesis is based on the assumption that PtdEtn is the only lipid species capable of being translocated. Since fluorescamine labeling can only determine the status of the aminophospholipids, the possibility of other lipid movements cannot be eliminated. It is possible that the outward flux of lipids is independent of the nature of the head group, while the inward movement is specific to the aminophospholipids, i.e., an in-to-out movement of lipids could occur in a lipid-species-independent manner. This outward translocation is slower compared with the rapid movement of the aminophospholipids to the inner leaflet. This would lead to a situation in which lipids are continuously, though slowly, translocated to the outer leaflet, while the aminophospholipids are rapidly and specifically translocated to the inner leaflet, leading to a steady-state condition in which they are present at relatively high concentrations in the inner monolayer. Such a mechanism has been shown to exist in the human-erythrocyte plasma membrane [46, 47]. The inability of fluorescamine to label detectable levels of PtdSer in normal and modified yeast cells can probably be explained if pump 2 is considered to have a higher affinity for PtdEtn than PtdEtn. Such a differential affinity has been demonstrated in the erythrocytes [38]. However, due to the inability of fluorescamine to label more than ~38% of the total yeast-cell PtdEtn in digitonin-permeabilized cells, it would be premature to comment conclusively on the status of this lipid in the yeast plasma membrane.

The above hypothesis assumes that pump 2 exhibits a poor sensitivity to the ATPase inhibitors. The experiments presented, however, may not account for this assumption.

Mechanisms (ii) and (iv). Since the use of ATPase inhibitors does not apparently affect the inward movements, the alternative possibilities, mechanisms (ii) and (iv), must be considered. According to these mechanisms, the in-to-out PtdEtn movements are N-ethylmaleimide sensitive, but may or may not be energy dependent, while the out-to-in translocation is neither N-ethylmaleimide sensitive nor energy dependent. Such an assumption would mean that the lipid movements are brought about by either simple diffusion, facilitated diffusion, a transmembrane pH gradient, a transmembrane potential gradient or asymmetrically polarized membrane proteins. These possibilities are intriguing for the following reasons.

As observed by Devaux [4], simple-diffusion and facilitated-diffusion models for the transbilayer lipid movements can account for the movement of phospholipids in the direction of the concentration gradient, such that an equilibrium state is reached in which each lipid species is equally distributed between the two leaflets. However, both the models fail to account for the movement of lipids against the concentration gradient, as observed here in yeast cells.

The influence of a transmembrane pH gradient on the regulation of the observed selective localization of phospholipids can be eliminated based on the observations of Cullis and coworkers who, by means of large unilamellar vesicles, have shown that only lipids that exhibit weak acid characteristics, such as free fatty acids, phosphatidylglycerol (PtdGro) and phosphatidic
acid, can cross the bilayer under the influence of a pH gradient [48–51]. The same workers have further shown that PtdSer does not flip in large unilamellar vesicles under the influence of a potential gradient [48]. Since the interior of the yeast cell has a pH of approximately 7, an acid, can cross the bilayer under the influence of a pH gradient [48]. Potential gradient in itself cannot bring about transbilayer movements of zwitterionic lipids, such as PtdEtn and PtdCho (movements due to a compensatory-flux mechanism in response to anionic lipids in the bacterial plasma membrane) [52, 53]. Thus, the proton gradient plays a very important role in the mechanisms of lipid flip-flop can also interfere with the modifications induced in the yeast cell in an attempt to study the communication no. 008/96 from Institute of Microbial Technology, Chandigarh, India.

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