The Proton Electrochemical Gradient across the Plasma Membrane of Yeast Is Necessary for Phospholipid Flip*  

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Recently, two members of the P4 family of P-type ATPases, Dnf1p and Dnf2p, were shown to be necessary for the internalization (flip) of fluorescent, 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled phospholipids across the plasma membrane of *Saccharomyces cerevisiae*. In the current study, we have demonstrated that ATP hydrolysis is not sufficient for phospholipid flip in the absence of the proton electrochemical gradient across the plasma membrane. This requirement was demonstrated by two independent means. First, collapse of the plasma membrane proton electrochemical gradient by the protonophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP) almost completely blocked NBD-phospholipid flip while only moderately reducing the cytosolic ATP concentration. Second, strains with point mutations in *PMA1*, which encodes the plasma membrane proton pump that generates the proton electrochemical gradient, are defective in NBD-PC flip, whereas their cytosolic ATP content is actually increased. These results establish that the proton electrochemical gradient is required for NBD-phospholipid flip across the plasma membrane of yeast and raise the question whether it contributes an additional required driving force or whether it functions as a regulatory signal.

The redistribution of phospholipids between the inner and outer leaflets of the plasma membrane of cells has been implicated in numerous cellular functions including membrane budding, vesicle fusion, and intercellular signaling (for recent reviews, see Refs. 1–4). Investigation of the mechanism of phospholipid redistribution across membranes and its role in these cellular functions has been stimulated by the identification of the P4 subfamily of P-type ATPases as candidates for the inwardly directed trans-bilayer transport (referred to as “flip”) of phospholipids across the plasma membrane (5). Members of this subfamily play a necessary role in the flip of phospholipids in several different species (6–8).

In *Saccharomyces cerevisiae*, it was recently shown that deletion of two members of the P4 subfamily, *DNF1* and *DNF2*, results in a significant decrease in the flip of NBD-labeled phosphatidylcholine, phosphatidylethanolamine, and phosphatidyserine (NBD-PC, NBD-PE, and NBD-PS) (8). Because P-type ATPases are known to couple the hydrolysis of ATP to the movement of ions across membranes (9) and ATP is required for phospholipid flip across the plasma membrane (8, 10), Dnf1p and Dnf2p, as well as other members of this subfamily, have been proposed to directly couple ATP hydrolysis to the transport of phospholipids (5, 8).

This interpretation is complicated by the observation that treatment of *S. cerevisiae* with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) almost completely blocks the flip of NBD-PC, NBD-PE (11, 12), and NBD-PS across the plasma membrane. Although CCCP collapses the mitochondrial proton electrochemical gradient in addition to that across the plasma membrane, yeasts grown in glucose do not depend on oxidative phosphorylation to produce ATP and would not be expected to have significantly reduced cytosolic ATP content (13). These data were interpreted to indicate that the plasma membrane proton electrochemical gradient is necessary for the flip of NBD-labeled phospholipids, even in the presence of normal levels of cytosolic ATP (11). Although the cellular ATP content was not measured, it was assumed to be maintained within normal limits and was required for the maintenance of the proton electrochemical gradient by the P-type ATPase Pma1p (11).

In this study, we measured the ATP content of cells following treatment with CCCP and observed that the concentrations of CCCP that almost completely inhibited the flip of NBD-PC only moderately reduced cytosolic ATP levels. Furthermore, in *pma1* mutant strains with reduced proton electrochemical gradients, we observed that NBD-PC flip was almost completely blocked, whereas the cytosolic ATP levels were actually increased. These data confirm a requirement for the proton electrochemical gradient across the plasma membrane for NBD-PC flip in yeast. This gradient may provide an additional driving force or a regulatory signal that is necessary for phospholipid flip in *S. cerevisiae*.

**EXPERIMENTAL PROCEDURES**

*Materials*—Yeast medium was purchased from Difco. NBD-PC was purchased from Avanti Polar Lipids. The ATP

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2 The abbreviations used are: NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBD-PC, 1-myristoyl-2-[6-(NBD)-aminocaproyl]-phosphatidylcholine; NBD-PE, 1-myristoyl-2-[6-(NBD)-aminocaproyl]-phosphatidylethanolamine; NBD-PS, 1-palmitoyl-2-[6-(NBD)-aminocaproyl]-phosphatidyserine; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

3 H. C. Stevens and J. W. Nichols, unpublished data.
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TABLE 1

Yeast strains used in this study

| Strain     | Genotype                      | Source                           |
|------------|-------------------------------|----------------------------------|
| LMY65      | HO arg6 pma1–105 (Ser-368→Phe)| J. McCusker laboratory           |
| LMY67      | HO arg6 pma1–141 (Ser-368→Phe)| J. McCusker laboratory           |
| LMY69      | HO arg6 PMA1                  | J. McCusker laboratory           |
| LMY94      | MATa leu2 METT15 lys2 ura3 his3| Laboratory collection*           |
| LMY94      | MATα leu2 METT15 lys2 ura3 his3[p'] | This study                    |

* From the J. W. Nichols laboratory collection.

an assay kit was purchased from Cambrex, Inc. Unless otherwise noted, all of the other materials were purchased from Sigma-Aldrich.

Yeast Strains and Culture—All strains used in this study (Table 1) were either from our laboratory collection or pma1 point mutant strains kindly donated by John McCusker (14). pΔ petites, which lack mitochondrial DNA, were made by treatment with ethidium bromide at 25 μg/ml in SDC synthetic complete medium (0.67% yeast nitrogen base, 2% glucose, and complete amino acid supplements) (15). Cells were grown overnight in SDC containing ethidium bromide, diluted 1:100 in the same medium and regrown overnight. The pΔ phenotype was tested by growth on glucose plates and lack of growth on glycerol plates. For all of the experiments, cultures were grown overnight at 30 °C in YPD (2% dextrose, 2% peptone, 1% yeast extract) diluted to A600 = 0.1 in SDC and grown to A600 = 0.2–0.4 prior to performing experiments.

Internalization of Phospholipids into Yeast Cells—NBD-PC internalization by yeast was performed as previously described (11). Briefly, NBD-PC was dried under a stream of nitrogen and resuspended in dimethyl sulfoxide at 1.25 mM. 2 μl of NBD-PC was added to early log phase cultures (A600 = 0.2–0.4) in 0.5 ml of SDC for a final concentration of 5 μM NBD-PC. Cells were vortex-mixed and then incubated on ice for 1 h. Afterward, cells were harvested and washed three times with two volumes of ice-cold SC azide (SDC lacking glucose but containing 2% sorbitol and 20 mM sodium azide) to prevent lipid efflux.

Flow Cytometry—Samples were analyzed via flow cytometry (FACScanIbar cytometer; BD Biosciences) as previously described (16). To detect and gate out dead cells, 10 μl of propidium iodide (50 μg/ml) was added to ~4 × 10⁵ NBD-PC-labeled cells in ~100 μl of SC azide immediately prior to analysis. Readings were taken on the flow cytometer equipped with an argon laser at 488 nm. Cell Quest software (BD Biosciences) and FlowJo (Tree Star, Inc.) were used to analyze and quantify the data. For comparison of parent cells to mitochondrial petite cells (pΔ), gating was performed for the analysis between forward scatter of 150–750 to ensure that similarly sized cells were compared.

Drug Treatment—For some experiments, cells were treated with CCCP prior to labeling. CCCP was resuspended in ethanol (5 mM stock) and added at various μM concentrations to the cells for 10 min at room temperature (30 min at 30 °C for pΔ experiment) before NBD-PC internalization assay or ATP assay was performed.

ATP Assay—A commercial ATP assay kit was purchased from Cambrex, Inc. and used in conjunction with ATP standards purchased from Sigma-Aldrich. 100 μl of CCCP- or etha-
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May inhibit the activity of any putative flipases. To test this possibility, we treated wild type cells in buffered SDC medium at pH values of 5, 6, and 7 with 50 μM CCCP and found no difference in NBD-PC flip (data not shown). Thus, we concluded that the effect of CCCP was a result of its collapse of the proton electrochemical gradient and not cytosolic acidification.

The Effect of CCCP Is Not Mediated by Collapse of the Mitochondrial Membrane Potential—In addition to inhibition of ATP synthesis, collapse of the mitochondrial proton electrochemical gradient by CCCP may activate a signaling pathway that down-regulates flip activity at the plasma membrane. Mitochondrial depolarization resulting from a loss of mitochondrial DNA has been shown to generate a retrograde signal to the nucleus that regulates genes involved in drug resistance (19). Although the rapid effect of CCCP is not likely to be mediated by transcriptional regulation, an acute signaling pathway may exist that senses mitochondrial membrane depolarization and down-regulates phospholipid flip activity at the plasma membrane.

To determine whether this hypothetical signaling pathway is necessary to mediate inhibition of phospholipid flip, we measured the effect of CCCP on NBD-PC flip in ρ0 cells. These cells are devoid of mitochondrial DNA and, as a result, have a defective electron transport chain. Thus, ρ0 cells cannot reduce NADH and FADH equivalents to produce a proton electrochemical gradient across the mitochondrial membrane, but they do maintain a normal gradient across the plasma membrane.

The results (Fig. 2) indicate that the ρ0 cells have slightly reduced NBD-PC flip compared with their isogenic parent cells but still responded to CCCP by decreasing NBD-PC flip by ~90%. The slightly reduced NBD-PC flip in the untreated ρ0 cells may result from the up-regulated retrograde signaling pathway that is known to increase the efflux of amphiphilic drugs and may also down-regulate influx (19). The almost complete inhibition of NBD-PC flip by CCCP argues that a signal resulting from the collapse of the mitochondrial proton electrochemical gradient is not necessary for the inhibition of NBD-PC flip across the plasma membrane by CCCP. This does not rule out the possibility that such a signaling pathway exists in normal mitochondria, but does rule out a requirement for this collapse to signal the almost complete block of NBD-PC flip following CCCP treatment. Thus, we concluded that the effect of CCCP on NBD-PC flip does not result from either a collapse of the mitochondrial proton electrochemical gradient or from an acute signal emanating from the mitochondria.

Mutations in PMA1 That Reduce the Plasma Membrane Proton Electrochemical Gradient Inhibit NBD-PC Flip and Increase Cellular ATP—To gain further insight into the relationship of the plasma membrane proton electrochemical gradient to phospholipid flip, we assayed NBD-PC flip in a strain carrying a mutation in the gene encoding Pma1p. Pma1p is also a member of the P-type ATPase family. It is essential because it electrogenically pumps protons out of the cell to create the proton electrochemical gradient across the plasma membrane that is required for ionic homeostasis and the uptake of nutrients (20). Because it is necessary for growth, mutations in PMA1 that cause a partial defect in pumping activity have been isolated and studied (21). One particular mutation, which was independently isolated in two mutant strains, pma1–105 and pma1–141 (14), caused a 65% reduction in ATPase activity and an inability to maintain an intracellular pH required for growth when incubated in acidic medium (21). These results were interpreted to reflect a reduced ability to pump protons from the cells (21). By comparing NBD-PC flip activity and cellular ATP levels in the pma1–105 and pma1–141 mutant strains relative to their isogenic parent strain, we could test the effect of reducing the proton electrochemical gradient across the plasma membrane without affecting the mitochondrial potential or reducing cellular ATP. Thus, the effect of decreasing the plasma membrane electrochemical proton gradient on NBD-PC flip could be tested in the absence of confounding effects that might arise from depolarization of the mitochondrial membrane potential.

The results (Fig. 3) indicated that the uptake of NBD-PC was reduced 75–80% in these mutant strains, whereas the amount of cellular ATP actually increased 2–2.5-fold. Cellular ATP increased presumably because of the reduced activity of Pma1p, which is a major consumer of cellular ATP (20). Conversely, an increase in Pmalp activity is the probable cause of the observed decrease in cytosolic ATP following collapse of the proton electrochemical gradient by CCCP treatment (Fig. 1).

The data in Fig. 3 show that, even in the presence of excess cellular ATP, the flip of NBD-PC is decreased in the PMA1 mutant strains due to a presumed dependence on the activity of Pma1p to generate a proton electrochemical gradient across the plasma membrane. We concluded from these data that ATP hydrolysis is not sufficient to drive the flip of NBD-PC across the plasma membrane in the absence of a plasma membrane proton electrochemical gradient.

DISCUSSION

The simplest model to explain the requirement of the P-type ATPases Dnf1p and Dnf2p for NBD-labeled phospholipid flip (8) is one in which the energy released from ATP hydrolysis is
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FIGURE 3. NBD-PC flip is reduced in pma1 mutant cells, whereas ATP levels are increased compared with isogenic parent. pma1 cells that were known to exhibit a decreased proton pumping activity were labeled with NBD-PC in early log phase (A600 = 0.2–0.4) for 1 h on ice or at 30 °C. NBD-PC internalization was quantified via flow cytometry and compared with isogenic parent cells. A small aliquot (100 μl) of these cells was removed before labeling, and ATP levels were measured via a luminometer and compared with parental levels. The parent cell ATP value, to which the data were normalized, was 1.15 × 10−2 ± 8.72 × 10−4 μmol of ATP/cell. pma1–105 and pma1–141 are different mutagenic isolates with the same Ser-368→Phe mutation. Data presented here are the mean ± S.D. of three independent experiments.

The results presented here do not directly address the role of ATP in the flip of NBD-PC but do establish an additional requirement (the proton electrochemical gradient) that must be incorporated into the molecular model describing flippase activity. This conclusion is supported by the experiments summarized below. First, we demonstrated that cells treated with CCCP show a strong inhibition of NBD-PC flip at the plasma membrane, although the ATP content of these cells was only modestly reduced (Fig. 1). Thus, the inhibition of NBD-PC flip by the collapse of the proton electrochemical gradient is not the result of reduced cellular ATP content. An indirect effect of CCCP on the mitochondrial proton electrochemical gradient was ruled out as the cause of reduced NBD-PC flip by showing that CCCP treatment blocked NBD-PC flip in ρ− cells that have a reduced mitochondrial potential (Fig. 2). In addition, CCCP treatment of cells previously labeled with NBD-PE (11) or NBD-PC (data not shown) does not alter the distribution of intracellular fluorescence. Thus, accumulation of NBD-PC and NBD-PE in the mitochondria is not dependent on the mitochondrial proton electrochemical gradient. From these experiments, we conclude that the inhibition of NBD-PC flip by CCCP does not result secondarily from a lack of its accumulation in mitochondria. The possibility that NBD-PC flip activity was inhibited by an increase in the intracellular pH resulting from CCCP treatment was ruled out by showing that the same inhibition was obtained in medium buffered at pH 5 as at pH 7. Furthermore, we demonstrated that mutations in the gene encoding Pma1p that partially disabled its ability to generate the proton electrochemical gradient across the plasma membrane also inhibited NBD-PC flip. Although methods are not currently available to measure the flip of endogenous phospholipids in yeast, inhibition by CCCP is not unique to the NBD-labeled phospholipids, because the flip of several BODIPY-labeled phosphatidylcholine analogues are inhibited by CCCP, even though they are flipped by different mechanisms than NBD-PC (12). Taken together, these data argue that, in addition to ATP hydrolysis, the plasma membrane proton electrochemical gradient is necessary for NBD-PC flip across the plasma membrane.

The role of the plasma membrane proton electrochemical gradient for phospholipid flip is unclear at this time. It could provide an additional contribution to the driving force for phospholipid flip across the bilayer. The energy gained from the hydrolysis of ATP may be insufficient in the absence of a favorable proton electrochemical gradient to overcome the high activation barrier for movement of a large hydrophilic headgroup through the hydrophobic bilayer interior. On the other hand, a reduced proton electrical chemical gradient may produce a signal that down-regulates phospholipid flip. One example of an alternative function of a P-type ATPase is the recent identification of a pool of sodium/potassium ATPase molecules that does not pump ions but instead binds Src kinase and regulates its activity (28). The possibility that P-type ATPases have signaling functions in addition to ion pumping activity opens the possibility that more complex models will be required to explain their role in phospholipid flip and membrane asymmetry.

Determining the role of the plasma membrane proton electrochemical gradient and its relationship to the yeast P4 subfamily of P-type ATPases in the movement of phospholipids across the plasma membrane remains a challenge to understanding the functional significance of the asymmetrical distribution of phospholipids across the plasma membrane.

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