Patch Clamp Studies on V-type ATPase of Vacuolar Membrane of Haploid Saccharomyces cerevisiae

PREPARATION AND UTILIZATION OF A GIANT CELL CONTAINING A GIANT VACUOLE

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A method for obtaining giant protoplasts of Escherichia coli (the spheroplast incubation (SI) method: Kuroda et al. (Kuroda, T., Okuda, N., Saitoh, N., Hiyama, T., Terasaki, Y., Anazawa, H., Hirata, A., Mogi, T., Kusaka, I., Tsuchiya, T., and Yabe, I. (1998) J. Biol. Chem. 273, 16897-16904) was adapted to haploid cells of Saccharomyces cerevisiae. The yeast cell grew to become as large as 20 μm in diameter and to contain an oversized vacuole inside. A patch clamp technique in the whole cell/vacuole recording mode was applied for the vacuole isolated from osmotic shock. At zero membrane potential, ATP induced a strong current (as high as 100 pA; specific activity, 0.1 pA/pM 3) toward the inside of the vacuole. Bafilomycin A 1, a specific inhibitor of the V-type ATPase, strongly inhibited the activity (K ) = 10 nM). Complete inhibition at higher concentrations indicated that any other ATP-driven transport systems were not expressed under the present incubation conditions. This current was not observed in the vacuoles prepared from a mutant that disrupted a catalytic subunit of the V-type ATPase (RH105(Δtma1::TRP)). The K value for the ATP dose-response of the current was 158 μM and the H/ATP ratio estimated from the reversible potential of the V-I curve was 3.5 ± 0.3. These values agreed well with those previously estimated by measuring the V-type ATPase activity biochemically. This method can potentially be applied to any type of ion channel, ion pump, and ion transporter in S. cerevisiae, and can also be used to investigate gene functions in various organisms by using yeast cells as hosts for homologous and heterogeneous expression systems.

For the evaluation of ion transport systems, the patch clamp method developed by Nehr and Sackman (1) in 1976 is one of the most direct and quantitative assay techniques which can be conducted under conditions similar to those in vivo. This technique, therefore, could potentially be one of the most powerful assay tools for the identification of transporter genes as well. Some transporter genes have been identified by introducing them into a heterogeneous expression system of Xenopus oocyte as a host for patch clamp recording (or two-electrode voltage clamp recording). Those genes had to be ones that Xenopus oocyte cells did not express or scarcely expressed on the Xenopus genome. For example, the genes for neurotransmitter receptor channels (2, 3) and carrier-type ion transporters such as the Na+/Ca2+ antiporter (4) were successfully identified using this particular method.

Since the gene manipulation technique can be applied much more easily to eukaryotic unicellular yeast cells than to animal cells, Kung and associates (5) first tried to identify some transporter genes in Saccharomyces cerevisiae. Thus far, only one gene of an outward-rectifying K+ channel, TO K1 (=DUK1= YKC1= YORK, Refs. 6–12) has been identified. For successful, quantitative patch clamp assays of ion pumps and carrier-type transporters that do not accompany high ionic currents, the sizes of the protoplasts are crucial; the small size of the cell used for the above study allowed identification only of ion channels with high ionic currents. For that reason, Bertl and co-workers (13) tried to prepare yeast protoplasts as large as 20 μm in diameter by digesting the cell wall using enzymes followed by prolonged incubation in an osmotically protective medium containing 200 mM KCl. It should be noted that they added no inhibitor of cell wall synthesis. They reported an ATP-induced current flow on the giant vacuole isolated from these oversized protoplasts (14). They appeared to have used polyploid cells, which were much larger than haploid cells to begin with, in order to obtain giant protoplasts as large as 20 μm. Polyploid cells, however, are not suitable for obtaining a disrupted mutant through genetic manipulation, because all the target genes on the multiple chromosomes have to be disrupted in order to obtain a stable phenotype.

Recently, we succeeded in converting Escherichia coli cells into giant protoplasts that are suitable for patch clamp experiments (15). This spheroplast incubation method (SI method)1 was a modification of the giant cell preparation method originally developed for Bacillus megaterium by Kusaka (16) in

1 The abbreviations used are: SI method, spheroplast incubation method; 2-DG, 2-deoxy-D-glucose; V-type ATPase, vacuolar proton-translocating ATPase; DTT, dithiothreitol; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, (3-morpholino)propanesulfonic acid; CD-CFDA, 5(and 6)-carboxy-2’,7’-dichlorofluorescein; PI, propidium iodide; f0, ohm.

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which giant protoplasts are formed after prolonged incubation of spheroplasts formed by treating cells with lysozyme in the presence of both penicillin G, an inhibitor of peptidoglycan synthesis, and an osmo-protectant.

We have decided to apply a similar technique to haploid cells of *S. cerevisiae*, and chose 2-deoxy-D-glucose (2-DG), a hydrophilic inhibitor of cell wall synthesis, since Biely and co-workers (17) found that a small amount of 2-DG, an analogue of glucose, specifically inhibits cell-wall synthesis without a significant effect on protein synthesis. In the present paper, we describe a method for preparing giant protoplasts of *S. cerevisiae* as large as 20 μm from haploid cells of the wild type and also a mutant that lacked the V-type ATPase activity. By using the whole cell/vacuole patch clamp technique upon giant vacuoles isolated from the giant cells by osmotic shock.

**FIG. 1.** Micrographs of giant cells from *S. cerevisiae* YPH500. A, a differential interference contrast (DIC) image of the giant cells. B, a magnified image of the area specified with the rectangular frame in A. C, a DIC image overlaid with a fluorescent image of a CDCFDA-stained giant cell. D, a phase contrast image of vacuoles isolated from the giant cells by osmotic shock.

**EXPERIMENTAL PROCEDURES**

Preparation of Giant Cells—The *S. cerevisiae* strains used were X2180–1A (MATa gal2 CUP1), YPH499 (MATa leu2 ura3 trp1 lys2 his3 ade2), and YPH500 (MATa leu2 ura3 trp1 lys2 his3 ade2). RH105 (Δvma1::TRP1 derivative of YPH500) was constructed as in Ref. 18. Innoculum from a stock culture on agar medium was grown in 4 ml of YPD medium supplemented with leucine (20 mg/liter), uracil (20 mg/liter), histidine (20 mg/liter), and adenine (20 mg/liter), since RH105 requires these nutrients for growth. Cells were all grown to about 10^8 cells/ml after this incubation. After 2 ml of 0.5 M KCl was added, the cells were harvested by centrifugation and resuspended in 2 ml of A medium. The suspension was divided into four 1.5-ml Eppendorf tubes (400 μl each) and stored at 10 °C until use. The stock can be stored for several hours without loss of activity. Prior to the next vacuole isolation step, the suspension was shaken for 3 h at 30 °C; spheroplasts had grown to 20–30 μm in diameter. The suspension was again harvested by centrifugation as described above, washed once with distilled water (supplemented with 1 mM DTT), and then resuspended in 2 ml of B buffer (1 M sorbitol, 1 mM DTT, and 0.1 M Tris-HCl, pH 7.2). Zymolyase was added to the suspension to give a final concentration of 1 mg/ml. The suspension was again shaken for 30 min. After confirming under a microscope that the cells had been fully converted to spherical cells (spheroplasts), they were harvested by centrifugation. The pellet was carefully resuspended in 2 ml of A medium (YPD medium supplemented with 1 M sorbitol and 0.05% 2-DG). One-hundred and fifty microliters of the suspension were diluted with 4 ml of A medium and incubated at 20 °C overnight on a shaker at 30 strokes/min. Since RH105, which is a catalytic subunit-disrupted mutant of the V-type ATPase, does not grow well at neutral pH but grows normally at lower pH (19), the YPD medium and the A medium were supplemented with 50 mM MES-MOPS to lower the pH to 5.5. These media were further supplemented with leucine (20 mg/liter), uracil (20 mg/liter), lysine (20 mg/liter), histidine (20 mg/liter), and adenine (20 mg/liter), since RH105 requires these nutrients for growth. Cells were all grown to about 10 μm after this incubation. After 2 ml of 0.5 M KCl was added, the cells were harvested by centrifugation and resuspended in 2 ml of A medium. The suspension was divided into four 1.5-ml Eppendorf tubes (400 μl each) and stored at 10 °C until use. The stock can be stored for several hours without loss of activity. Prior to the next vacuole isolation step, the suspension was shaken for 3 h at 30 °C; spheroplasts had grown to 20–30 μm by this time. Two-hundred microliters of 0.5 μm KCl were added to each tube, which was then centrifuged in an Eppendorf-type centrifuge at 5,000 rpm for 5 min. The pellet was washed in 1 ml of C buffer (A buffer supplemented with 0.8 M sorbitol and 0.1 μM KCl), and incubated for 10 min at 30 °C on a shaker at 30 strokes/min. The cells were harvested as described above, resuspended in 1 ml of D buffer (0.8 M sorbitol, 0.1 μM KCl, 1 mM DTT, and 0.1 M Tris-HCl, pH 7.2) supplemented with 1 mg/ml Zymolyase, and then incubated for 30 min at 30 °C on a shaker at 30 strokes/min. Microscopic observation confirmed that the cells were fully converted to spheroplasts.

**Microscopy and Electron Microscopy**—For vacuole staining, spheroplasts were incubated in the YPD medium supplemented with 1 M sorbitol, 50 mM citric acid, and 10 μM CDCFDA (20), and were shaken.
at 30 strokes/min for 30 min at 30 °C. A vacuole was observed as a bright yellow fluorescent sphere inside the cell. This result indicates that the CDCCFDA added to the medium was transported through the cytoplasmic membrane into a vacuole, hydrolyzed by an esterase, and became the fluorescent in the acidic vacuolar lumen. For nuclei staining, propidium iodide (PI) was used as fluorescent dye according to Ref. 21. A confocal laser scanning microscope (TCS4D, Leica Co.) was used for observation with excitation at 488 nm. For CDCCFDA and PI, a 550-nm band path filter and a 550-nm long path filter were used for emission, respectively. For electron microscopy, giant cells (X2180-1A) were fixed by using a conventional glutaraldehyde/OsO4 method without Zymolyase treatment (22); for intact cells (YPH499), a freeze-substitution method (23) was used. Ultrathin sections were stained with uranyl acetate and Reynolds’s lead citrate, except for the observation of cell wall, for which a silver proteinate method for carbohydrate staining (24) was employed.

Isolation of Vacuoles—The suspension of giant spheroplasts obtained above was concentrated 5-fold by centrifugation. Ten microliters of the concentrate were transferred to the recording chamber of the patch clamp apparatus, diluted there with 200 μl of E buffer (0.1 M sorbitol, 0.1 mM KCl, 5 mM EGTA, 20 mM Tris-MES, pH 7.5), and allowed to stand for 5 min. Under the microscope, it was observed that more than half of the cells were broken and released vacuoles into the medium. Vacuoles of more than 20 μm were selected for patch clamp experiments. As released vacuoles tend to stick to the glass wall of the chamber, broken spheroplasts, broken cytoplasmic membranes, organelles, and other debris can easily be washed away by pouring F buffer (0.1 M sorbitol, 0.1 mM KCl, 2 mM MgCl2, 1 mM EGTA, 0.15 mM CaCl2 (10 mM free Ca2+), and 10 mM Tris-MES, pH 7.5) through a capillary tube with six-way valves as described below.

Patch Clamp Recording—Experiments were performed basically as described by Hamill et al. (25). Capillaries were made of 75-μl disposable glass micropipettes (Duramont, Bromall, PA) using a two-stage pulling apparatus (PP-83, Narishige, Tokyo), and the tips were heat-polished. The conductivity of the open capillaries filled with G buffer (0.1 M sorbitol, 0.1 mM KCl, 2 mM MgCl2, 10 mM MES-Tris, pH 5.5, 2 mM CaCl2) ranged from 3 to 5 MΩ. To produce a whole cell/vacuole patch, after a tight seal was formed (10 GΩ, the patched part of the vacuole membrane was broken by applying a few pulses (<2.0 volts) with duration ranging from 1 to 10 ms. After that, the resistance became 1 to 5 GΩ. Five minutes were usually long enough to exchange the medium inside the vacuole with another medium through the capillary by diffusion. The external medium was changed by a tandem 6-way valve system as described previously (15). Membrane currents were amplified by a patch/whole cell clamp amplifier (CEZ-2300, Nihon Kohden Co., Tokyo) and recorded on a digital audio tape recorder (DTC 55ES, Sony Corp., Tokyo). Stored data were subsequently processed and analyzed by using a personal computer (PC-9801DX, NEC Inc., Tokyo) and a software program (QP-120J, Nihon Kohden Co., Tokyo). Sign conventions throughout this report define the vacuolar interior as Ref. 26, so that positive membrane voltages mean that the cytoplasmic electric potential is positive to the vacuolar potential, and a positive current represents positive charge moving from the cytoplasm to the inside of a vacuole. Experiments were performed at room temperatures (20–23 °C).

Reagents—Peptone and yeast extract were purchased from Difco; sorbitol, EGTA, and DTT from Sigma; 2-deoxy-D-glucose from Wako Pure Chemicals, Osaka; CDCCFDA, from Molecular Probes Ltd.; Zymolyase (20T) from Seikagaku Kogyo Co Ltd., Tokyo. Other reagents were all of analytical grade.

RESULTS

Morphology of Giant Cell—As shown in Fig. 1, A and B, the haploid cells of S. cerevisiae were all converted to giant cells as large as 20 μm, five times larger than untreated cells. Most of these cells were spherical; some had buds that appeared to have stopped developing further. In order to confirm that the spherical cells were protoplasts without the cell wall, electron microscopy was conducted (Fig. 2). The magnified images of the cell envelope revealed that the cells (protoplasts) lacked cell wall carbohydrates that would be stained by the silver proteinate method (Fig. 2C). The envelope of intact cells was well stained (27) and showed a thick layered structure with β-glucan and mannann-protein layers (Fig. 2D). Further treatment with Zymolyase resulted in total conversion of the cells into the spherical shape. The ratio of 2-DG/glucose was found to be critical: ratios neither lower nor higher than 1/40 were effective in producing the giant protoplasts. The otherwise normal growth indicates that 2-DG at this critical concentration does act as an inhibitor of cell wall synthesis but does not significantly affect energy-generating glycolysis (17). It appears that the yeast cells were converted to giant protoplasts merely by the inhibition of cell wall synthesis. Although much needs to be done to elucidate the mechanism, fluorescence microscopy of the cells stained with PI for DNA (Fig. 3) may provide some insight into this problem. These pictures show that the cells became multinuclear when budding was arrested either in the middle (Fig. 3, A and B) or at the start (Fig. 3, C and D). These observations are consistent with previous results with a mutant cell in which the cell morphogenesis checkpoint system was genetically impaired: nuclear divisions continued while the corresponding synchronous budding cycle (cytoplasmic division) was halted (28, 29). It should be emphasized that these giant protoplasts, although they have multiple nuclei, derive originally from haploid cells and thus have identical nuclei.

Ion Transporters on Giant Vacuole Membrane—As seen in a micrograph (Fig. 1B), the giant protoplast was occupied by a huge organelle that appeared to be a vacuole. This organelle was indeed confirmed to be a vacuole, since it was stained with a vacuole-specific CDCCFDA (Fig. 1C) and did not show any electron-dense material inside the vacuole in an ultrathin sec-
tion under the electron microscope (Fig. 2A). By slightly lowering the osmotic pressure of the medium, the cell membrane of the giant protoplast was broken and, as a result, the intact vacuole was readily released into the medium (Fig. 1D). The vacuole thus released swelled up and became as large as 30 μm, adhering well to the surface of the glass slide along with small pieces of broken cytoplasmic membrane and small spheroidal bodies acting like glue. The adhesion was stable enough for buffer washing through the capillary, thus enabling quick exchange of the medium simply by using the six-way valve system.

The patch clamp technique in the whole cell/vacuole recording mode was applied to this vacuole. Two types of ion-transporter activities were detected. Fig. 4 shows an anion-specific transporter. With equimolar potassium (100 mM) on both sides of the membrane, the voltage versus current curve (V-I curve) changed little when 90% of the anion outside was replaced with fluoride (open circles); on the other hand, glutamate (triangles) and nitrate (squares) drastically changed the curve. With the Goldman equation and using the reversal potentials of these anions, permeability ratios were calculated to be 2.8 ± 0.2:1.05 ± 0.3:0.1 ± 0.015 for NO₃⁻:Cl⁻:F⁻:glutamate⁻. These results are consistent with previous reports that indicate that there is an electrogenic anion transporter on the vacuole membrane (30), and that glutamate is accumulated mainly in the cytoplasm (31) and nitrate in the vacuole (32).

A distinct ion-transport activity is shown in Fig. 5. When 1 mM ATP was poured onto the surface of the vacuole through the capillary with the membrane potential being kept at zero, a huge current as high as 100 pA (0.1 pA/μm²) flowing into the vacuolar lumen was observed (Fig. 5A). After a transient overshoot, the current gradually reached a steady-state level. Washing the medium outside the vacuole with an ATP-free medium brought the current down to the baseline level. Bafilomycin A₁, which is a potent and specific inhibitor of V-type ATPase (33), strongly inhibited this ATP-induced pump current (Fig. 5B). At 10 μM, the pump was completely inhibited (data not shown); 50% inhibition was achieved at 10 nM. In order to confirm that this ATP-induced current is generated by V-type ATPase, mutant cells that lacked the V-type ATPase activity were also converted to giant cells using the SI method. The RH105 strain (Δvma1::TRP) was cultivated and converted to giant protoplasts in a similar way, except that the
pH was kept at 5.5 as stated under “Experimental Procedures.” As expected, while no significant difference was observed in terms of the above described anion-specific transporter activity between the wild type (YPH500, Fig. 6B) and the mutant (RH105, Fig. 6C), the ATP-induced current was detected only in the wild type and could not be detected in the RH105 mutant (Fig. 6A). It was thus confirmed that no other ATP-dependent pump such as Ca^{2+}-ATPase (34) was induced under our present experimental conditions.

It should be noted here that in this particular experiment (Fig. 6A) an ATP-regenerating system consisting of creatine phosphate and creatine kinase was supplemented in the medium in order to minimize the ADP concentration, because ADP is known to inhibit the activity (35). As a result, the overshoot observed in earlier experiments (Fig. 5) no longer appeared. Experiments hereafter were all conducted under these conditions.

**Properties of the ATP-induced Current Due to V-type ATPase**—The ATP-induced current was measured at different ATP concentrations (Fig. 7A). A double reciprocal plot of the current against the ATP concentration is shown in Fig. 7B. The apparent $K_m$ value for ATP was calculated to be 0.159 mM, which is in good agreement with the $K_m$ value (0.2 mM) obtained by measurements of ATP hydrolysis for the yeast V-type ATPase (36).

Since the two V-I curves, one for the ATP-induced current and the other for the ATP-independent current (Fig. 8A) included a component due to the anion-specific transporter and a small leak current, the subtracted difference was plotted in Fig. 8B in order to obtain the “true” V-I curve for the ATP-dependent pump. This curve has two distinct characteristics: (a) the $H^+$-pump activity saturates at sufficiently high membrane potentials (>40 mV); (b) it does not become negative even at sufficiently low potentials (<70 mV). The former observation (a) suggests that the $H^+$-pump is accelerated with an increase of the membrane potential until the rate becomes limited by a step independent of the potential such as ATP hydrolysis. The latter (b) indicates that the $H^+$-pump (V-type ATPase) does not work in the negative direction which is necessary, if any, for the synthesis of ATP. This is consistent with the previous result that indicated that the V-type ATPase has no ATP-synthase activity (37).

Based on the reversal potential of −70 ± 5 mV and $\Delta$pH of 2.0 units, the electrochemical potential difference of $H^+$ ($\Delta$mu$_{H^+}$) was calculated to be −190 ± 5 mV, which is very close to the value obtained with vacuolar membrane vesicles isolated from normal size yeast cells (36). The free energy difference of ATP in equilibrium with this system ($\Delta G_{ATP}$) = −15.3 Kcal/mol) was calculated by adopting a value for $\Delta G_{ATP} = −9$ Kcal/mol (38) and RT In (ADP/[P]/[ATP] = −6.3 Kcal/mol, which was estimated from the equilibrium constant for the creatine kinase reaction (39). From the reversible $\Delta$mu$_{H^+}$ and $\Delta G_{ATP}$, the $H^+$/ATP ratio was estimated to be 3.5 ± 0.3. In a previous report, the $H^+$/ATP ratio for a plant vacuolar $H^+$-pump was directly calculated to be 2 from the amounts of transported $H^+$ and hydrolyzed ATP (40), which has since been assumed to be a fixed value. A recent result from patch clamp experiments with the vacuole of a plant cell, where inside and outside pH was widely varied, showed that the $H^+$/ATP ratio varied from 1.75 to 3.28 depending on the pH inside and outside of the vacuole (41). When the outside medium was basic (pH 8.0) and $\Delta$pH was large (4.7), the $H^+$/ATP ratio was 1.75; when the outside medium was neutral (pH 7.0) and $\Delta$pH was small (2.2), the ratio was as high as 3.28. The present $H^+$/ATP ratio was close to the latter. The pH conditions of the present experiments were similar as well. We have not measured the ratios under different pH conditions yet, for which a series of experiments are in progress at the moment.

Our present experiments, in which the patch clamp method was applied to a giant yeast vacuole prepared by using the SI method, were quite reproducible, and enabled us to elucidate most of the in vivo biochemical properties of the yeast V-type ATPase. In the previous studies on the in vitro activity of the yeast V-type ATPase (35, 36), more than 100 g of batch-cultured cell pellets had been needed. Moreover, this method was found to be potentially useful for analyzing the reversibility of the $H^+$-pump and its membrane potential-dependent regulatory mechanism. The general features of the present whole cell/vacuole patch method is thus applicable to a number of studies on vacuolar and lysosomal functions in many other organisms, simply by employing S. cerevisiae vacuoles as host organelles.

**DISCUSSION**

Kusaka in 1967 (16) used penicillin G as the cell wall synthesis inhibitor in his original SI method for preparing giant protoplasts of B. megaterium. Penicillin G was also effective for E. coli (15). However, no hydrophobic reagent like penicillin G is known for yeast that inhibits cell wall synthesis (42) but does not affect other cellular functions, particularly, transport activities in the membrane. Bielew and co-workers (17) found that a low concentration of 2-DG, a glucose analogue, specifically inhibited cell wall synthesis without significantly interfering with protein synthesis. They assumed that 2-DG was phosphorylated to 2-deoxyglucose-6-phosphate, which could not enter
the glycolytic pathway and instead entered the pathway for structural polysaccharide synthesis and in turn inhibited one of the intermediate steps (43, 44). In the present work, we chose this glucose analogue and found that, at its critical concentration, 2-DG was quite effective in forming giant protoplasts from haploid cells of *S. cerevisiae*.

We showed that by using a (modified) SI method, a haploid cell of *S. cerevisiae* could be converted into a giant protoplast that contained a single vacuole as large as 20 μm. The giant vacuole was readily released in a free form and could be used for the whole cell/vacuole mode patch clamp measurement. The H^+-pump was quantitatively analyzed and identified as the V-type ATPase that had been studied biochemically (35, 36). We have recently succeeded in whole cell patch clamp recordings of the yeast giant protoplasts as well. Thus, a combination of these two methods together shall provide a powerful system for analyzing any ion transporter, not only highly active channels (5–12, 45–48) but also other weaker transporters in the membranes, either vacuolar or cytoplasmic, of *S. cerevisiae*. We used haploid cells because they are much easier than polyploid ones to manipulate genes to yield deletion mutants. Recently, Bertl and co-workers (14) reported a method for preparing a giant protoplast from *S. cerevisiae* polyploid cells. By using a patch clamp recording, they subsequently revealed an ATP-induced pump activity on the vacuolar membrane. Its biochemical properties were less well understood (14).

The yeast vacuole is known to function as a temporary storage compartment and sequesters in it various metal ions (*e.g.* Ca^{2+}, Na^{+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+}, and K^+), basic amino acids, and phosphates. Cd^{2+} ions are taken up and detoxified inside the vacuole (49). Many of these transporters are likely to be exchange systems coupled with H^+ (34, 50–53) and largely driven by ΔμH^+ -- which is generated by the V-type ATPase (H^+-pump) (see details in Refs. 49 and 54–56). We show in this paper that the patch clamp method can quantitatively measure ion-transport activities and their physiological regulatory mechanisms under conditions very close to those in vivo. Ionic environments inside and outside of the membrane as well as the voltage can readily be changed for the analysis. This technique together with the giant vacuole preparation will certainly contribute to a further understanding of the vacuole transport network and ion homeostasis in the vacuole.

### Identification of Transporters from Yeast

The sequencing of genomic DNA has been completed in as many as 20 species including *E. coli* and *S. cerevisiae*. In microorganisms alone,
the sequencing of as many as 78 species is presently being undertaken. But even in *E. coli*, which seems to have perhaps been the most vigorously pursued among them, only 60% of the genes have been either functionally analyzed or assigned through an homology search (58). In the case of *S. cerevisiae*, only 43% have so far been identified (59). On the basis of amino acid sequence analyses that predict the existence of more than two transmembrane spans, 14% of the genes have somehow been speculated to be related to membrane transport (60). Less than 30% of these genes have so far been assigned.

In addition to these genomic data as well as the DNA microarray method (see Ref. 61, for review), our present SI method in combination with the patch clamp technique will certainly promote the targeting and identification of numerous yet to be identified genes for ion transport. A number of such projects are presently being pursued in our laboratory.

**Identification of Transporters from Plants and Animals—**

Several genes for ion transporters from plant and animal cells have been cloned by means of the transformation of recombinant expression vectors with cDNA libraries into appropriate yeast deletion mutants. *S. cerevisiae* is one of the best host cells for the introduction of other eukaryotic genes; haploid cells are easily manipulated for obtaining deletion and/or null mutants; the sequence of the entire genomic DNA has been determined; routine techniques for gene manipulation and analysis have been established; and the largest number of genes have been cloned and functionally analyzed among the eukaryotes. In plant cells, protein synthesis and targeting machinery are closer to yeast cells and more conserved than those of animals. Thus, plant genes are easily confirmed to functionally complement yeast genes (62, 63). In fact, approximately 20 ion transporter genes, e.g. AKT1 for K⁺ transporter, from plant cells have been cloned (64). From animal cells, however, ORK1 (K⁺ channel) is the only one that has been cloned so far (65).

For an electrophysiological assay of these ion transporters, direct measurements of the electric current by using either the two-electrode voltage-clamp method or the patch clamp method have been applied to appropriate host cells in which a target gene of interest has been expressed. In most cases, a heterologous expression system of *Xenopus* oocytes has been used to analyze functionally a large number of animal genes, e.g. nAChR for cation channel (2) and plant genes, e.g. KAT1 for K⁺ channel (66). Perhaps, the intrinsic drawback of *Xenopus* oocyte cells is the difficulty of making deletion mutants. It is essential for successful measurement to suppress the endogenous current that often interferes with measurement of a weak current due to the specific transporter under investigation. In the past, the background current was either suppressed using a specific inhibitor (nAChR cation channel, Ref. 2), or overcome by expressing a large amount of the transporter protein (Na⁺ channel, Refs. 67 and 68). For many other endogenous currents, however, one cannot always find specific inhibitors (69). In other cases, functionally similar endogenous transporters seem to be expressed more significantly than can be ignored (70).

The ultimate solution for the above mentioned problems would be the use of a host cell that has been deprived of genes responsible for the background current. Those cells that can readily be used for this purpose would be haploid cells of *S. cerevisiae* that are thus much easier to handle genetically than either *Xenopus* oocyte or baculovirus/insect cells. Despite these advantages, only a few studies have so far been reported; KAT1 from plant cells is one of the best examples (57). In this work, Bertl and co-workers (57) deleted the endogenous K⁺ transporter genes (TRK1 and TRK2) of *S. cerevisiae*, and thus suppressed the background current completely. With this cell, they quantitatively analyzed the inward rectifying current of a K⁺ channel due to KAT1. As stated above, several genes for ion transport systems from plant cells have been cloned. Many other genes could be future targets to be studied electrophysiologically. The present SI method established for haploid cells of *S. cerevisiae* will provide a potential advantage for studies on the molecular biological functions of channel/transporter genes.

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**REFERENCES**

1. Nehr, E., and Sakman, B. (1976) *Nature* **260**, 799–802
2. Mishina, M., Kurosaki, T., Tobimatsu, T., Morimoto, Y., Noda, M., Yamamoto, T., Tregos, M., Lindstrom, J., Takahashi, T., Kuno M., and Numa, S. (1984) *Nature* **307**, 604–608
3. Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M., and Nakanishi, S. (1987) *Nature* **329**, 836–838
4. Hilgenmann, D. W., Nicoll, D. A., and Philipson, K. D. (1991) *Nature* **352**, 715–718
5. Gustin, M. C., Matina, B., Saimi, Y., Culbertson, M. R., and Kung, C. (1986) *Science* **233**, 1195–1197
6. Ketchum, K. A., Joiner, W. J., Sellers, A. J., Kaczmarek, L. K., and Goldstein, S. A. N. (1995) *Nature* **376**, 690–695
7. Lesage, F., Guillemer, E., Fink, M., Duprat, F., Lazdunski, M., Romey, G., and Barhanin, J. (1996) *J. Biol. Chem.* **271**, 4183–4187
8. Zhou, X-L., Vaillant, B., Loukin, S., H. Kung, C., and Saimi, Y. (1996) *FEBs Lett.* **373**, 170–176
9. Reid, J. D., Lukas, W., Shaafastani, R., Bertl, A., Scheumann-Kettner, C., Guy, H. R., and North, R. A. (1996) *Recept. Channels* **4**, 51–62
10. Bertl, A., Bihler, H., Reid, J. D., Kettner, C., and Slayman, C. L. (1998) *J. Membr. Biol.* **162**, 67–80
11. Loukin, S. H., Vaillant, B., Zhou, X-L., Spalding, R. P., Kung, C., and Saimi, Y. (1997) *EMBO J.* **16**, 4817–4825
12. Vergani, P., Misoga, T., Jarvis, S. M., and Blatt, M. R. (1997) *FEBs Lett.* **405**, 337–344
13. Bertl, A., and Slayman, C. L. (1992) *J. Exp. Biol.* **172**, 271–287
14. Bertl, A., Bieler, H., Kettner, C., and Slayman, C. L. (1998) *Pflugers Arch. Eur. J. Physiol.* **436**, 999–1013
15. Kuroda, T., Okuda, N., Saitoh, N., Hiyama, T., Terasaki, Y., Anazawa, H., Hirata, A., Mogi, T., Kusaka, I., Tsuchiya, T., and Yabe, I. (1998) *J. Biol. Chem.* **273**, 16897–16904
16. Kusaka, I. (1967) *J. Bacteriol.* **96**, 884–888
17. Bieley, P., Kratky, Z., Kovařík, J., and Bauer S. (1971) *J. Bacteriol.* **107**, 121–129
Patch Clamp Studies on V-type ATPase of Vacuolar Membrane of Haploid Saccharomyces cerevisiae: PREPARATION AND UTILIZATION OF A GIANT CELL CONTAINING A GIANT VACUOLE

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