Patch Clamp Studies on Ion Pumps of the Cytoplasmic Membrane of Escherichia coli

FORMATION, PREPARATION, AND UTILIZATION OF GIANT VACUOLE-LIKE STRUCTURES CONSISTING OF EVERTED CYTOSPLASMIC MEMBRANE*

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The patch clamp technique is an excellent method to measure ion movement across cell membranes as current (1). An extremely small glass pipette (about 1 μm in diameter) is attached to the membranes, and activity of ion translocating proteins (ion channels, ion pumps, or ion transporters) is directly measured. So far, however, this important method has mainly been utilized for studies on animal or plant cells but scarcely for bacterial cells (2). Bacterial cells are usually too small to be measured by this method.

Escherichia coli, a Gram-negative bacterium, is the best characterized organism from both biochemical and genetical points of view. Ion pumps and ion transporters in E. coli are biochemically well characterized. Many mutant E. coli cells are available. Thus, genetical manipulations are very easy with this microorganism. Therefore, development of a patch clamp method applicable to E. coli membranes must be extremely valuable. Cells of E. coli are surrounded by an outer membrane and an inner membrane (cytoplasmic membrane) separated by a peptidoglycan layer and a periplasmic space. All of the major ion pumps and ion transporters such as the respiratory chain, F$_0$F$_1$-ATPase, various ion transporters, and ion-coupled solute transporters are located in the cytoplasmic membrane. To measure ion translocation via such ion pumps or transporters of the cytoplasmic membrane, we have to overcome the following three hurdles: 1) we have to prepare giant vesicles, the diameter of which must be at least 10 μm (this is important to get high success rate and accuracy of measurement), 2) the pipette must be directly accessible to the cytoplasmic membrane, and 3) the substrates or effectors of the ion pumps or transporters must be easily accessible to the active site of the proteins and easily removable from the system.

It would be essential to prepare giant protoplasts to overcome the first two hurdles. Many attempts have been made by many research groups to prepare giant bacterial cells or giant protoplasts. So far, however, no giant protoplasts surrounded by cytoplasmic membranes and suitable for patch clamp analysis have been prepared. Giant cells that were large enough for the patch clamp analysis have been prepared from cephalaxin-induced filaments (3) or from an osmotic-sensitive mutant (4). However, such cells were not protoplasts surrounded only by the cytoplasmic membrane. Attempts have been made to prepare giant protoplasts from such giant cells (or spheroplasts) by osmotic shock or by treating them with lysozyme (5, 6). It is not clear whether the respiratory chain components and F$_0$F$_1$-ATPase (H$^+$-translocating ATPase) are present in the membrane of such preparations, and therefore origin of the membrane is not clear. On the other hand, large protoplasts have been prepared from a penicillin-resistant mutant (7, 8). Those protoplasts were still too small to apply for the patch clamp method. Thus, these preparations were not suitable for the measurement of ion translocation via proteins of the cytoplasmic membrane by the patch clamp method. Recently we have succeeded in developing a unique method, named the spheroplast incubation method, for the preparation of extremely large giant protoplasts (10–30 μm in diameter). This method was derived from an early observation by Kusaka (9) that large protoplasts are formed after prolonged incubation of spheroplasts formed by treating cells of Bacillus megaterium with lysozyme in the presence of both penicillin G, an inhibitor of peptidoglycan synthesis, and an osmo-protectant. We have applied this method to many Gram-negative and -positive bacteria and succeeded in the formation of giant protoplasts from...
the cytoplasmic membrane of E. coli cells are available, the size of the vesicles is much smaller than the original cells (11, 12).

Formation of vacuole-like structures in E. coli cells has been reported from two groups. Lederberg and Clair reported that vacuole-like structures appeared in cells after incubation in the presence of penicillin G, which resulted in cell lysis (13). Buchner et al. observed vacuole-like structures in osmotic-sensitive mutant cells by ultra thin section electron microscopy (4). No ribosomes were observed in the vacuole-like structures. Both types of vacuole-like structures were about 3 μm. Unfortunately, further analysis of these vacuole-like structures has not been done. During the course of our studies on the giant pro-vacuoles, we found that extremely large vacuole-type structures (provacuoles) from E. coli cells. We investigated the properties of the giant provacuoles and measured H⁺ translocation via the respiratory chain (or by other bacteria).

**EXPERIMENTAL PROCEDURES**

**Preparation of Giant Protoplasts from E. coli Cells—** Cells of E. coli K002 (Lpp⁻) or K003 (Lpp⁺, ΔuncB-C::Tn10) (14) were grown in a rich medium, beef heart infusion broth (Difco Co.) or LB broth, containing suitable antibiotics under aerobic conditions. The cells were harvested in the late exponential phase of growth and suspended in the same volume of SP buffer (25 mM Tris-HCl, pH 7.4, and 400 mM sucrose). Lysozyme (200 μg/ml) was added to the cell suspension, and the suspension was shaken at 30 °C for 10 min at 45 rpm. After this treatment, the cells were harvested and resuspended in the same volume of the GP medium consisting of 2.75% trypticase soy broth (without dextrose) (BBL Co.), 10 mM MgSO₄, and 200 mM sucrose. A 1/100 volume of this suspension was diluted into the GP medium containing 0.7 units/ml of K002 (Lpp concentration was 300 mM). The plasmids constructed were designated as pMAL-pCM-GFP and pMAL-cCM-GFP. Expression of maltE gene carried on the plasmids is under the control of tac promoter, so that the protein was induced with IPTG.

**Preparation of Provacuoles from Giant Protoplasts—** Giant protoplasts (induced with IPTG when necessary) were harvested by centrifugation at 1,600 × g. The pellet was washed with Burst buffer consisting of 10 mM Tris- HCl, pH 7.4, 10 mM MgCl₂, 50 mM sucrose, 0.5 mM phenylmethylsulfonfony fluoride (PMSF), and 17.5 units/ml of DNase I. The suspension was shaken at 30 °C for 20–30 min (45 rpm). After centrifugation at 1,600 × g, the pellet was resuspended in a small volume of Burst buffer containing 20% Percoll (Amersham Pharmacia Biotech). The suspension was placed in a centrifuge tube onto which an equal volume of Burst buffer was overlaid. Percoll density gradient centrifugation was carried out at 400 × g for 30 min. Provacuoles were found in the interfacial layer. The giant provacuoles were washed twice with the Burst buffer and used for further analyses. Membranes of the provacuoles and intraprovacuolar materials were separated as follows. The giant provacuoles were frozen rapidly in liquid N₂ and thawed on ice. The provacuoles were completely disrupted by this procedure. After ultracentrifugation at 130,000 × g for 1 h, the pellet (membrane fraction containing the supernatant and the intraprovacuolar materials) was washed with Burst buffer. The membrane fraction was washed with Burst buffer and resuspended in the same buffer. All centrifugations were carried out at 4 °C. The fractions (membranes and intraprovacuolar materials) were used for detection of MBP.

**Microscopy and Electronmicroscopy—** For DAPI staining, the giant protoplasts were prepared from cells of E. coli K002. DAPI (final concentration, 1 μM) was added to the suspension of giant protoplasts and incubated at 30 °C for 2–3 h. When necessary, the giant protoplasts were isolated from the protoplasts. Fluorescent micrographs of the protoplasts and the provacuoles were taken with excitation at 350 nm and emission at 430–450 nm. For detection of GFP, the giant protoplasts were prepared from cells of E. coli C600/pMAL-pCM-GFP or C600/pMAL-cCM-GFP. IPTG was added to the medium at 0.1 mg/ml and incubated for 2–3 h. The fluorescent micrographs were taken by confocal laser scanning microscopy (TCS4D, Leica Co.) with excitation at 488 nm and emission at 530 nm. Electron micrographs were taken as described previously (15).

**Preparation of Membrane Fraction—** Intact cells of E. coli K002 were harvested by washing twice in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and 1 mM PMSF. The cells were disrupted by sonication. After removal of unbroken cells by low speed centrifugation, the supernatants were centrifuged at 100,000 × g for 1 h at 4 °C. Membrane fractions were recovered in the pellet fraction. The membrane fraction was washed with a buffer consisting of 10 mM Tris-HCl, pH 7.4, and 2 mM EDTA. Membrane fraction from the giant protoplasts was prepared as described above. The giant protoplasts were collected by centrifugation and suspended in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM PMSF, and 280 units/ml of DNase I. The giant protoplasts were completely disrupted by sonication. Unbroken protoplasts were removed by low speed centrifugation. The supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The pellet was washed as described above. The membrane fractions were resuspended in a buffer consisting of 25 mM Tris-HCl, pH 7.4, and 0.25% Sarkosyl and incubated at 20 °C for 20 min. Outer membrane proteins were not solubilized and could be obtained as pellets after centrifugation. The pellets were washed with a buffer consisting of 10 mM Tris-HCl, pH 7.4, and 5 mM MgCl₂ and resuspended in the same buffer.

**Detection of MBP by Western Blotting—** The giant protoplasts were washed twice with a stabilizing buffer consisting of 10 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 300 mM sucrose, 50 mM KCl, 0.5 mM PMSF, and 17.5 units/ml DNase I and resuspended in the same buffer. Giant vacuoles, the provacuolar membranes, and intraprovacuolar materials were prepared as described above. One-fourth volume of 50% tetrachloracetic acid was added to the samples and mixed vigorously. After centrifugation for 1 h, the supernatants were collected by centrifugation. The supernatants were diluted into the GP medium containing 0.7 units/ml of K002 (Lpp concentration was 300 mM). The plasmids constructed were designated as pMAL-pCM-GFP and pMAL-cCM-GFP. Expression of maltE gene carried on the plasmids is under the control of tac promoter, so that the protein was induced with IPTG.
transferred to a polyvinylidene fluoride membrane after polyacrylamide gel electrophoresis. Anti-SecY antiserum and the ECL system were used to detect the SecY. Anti-SecY antiserum and purified SecY protein were generous gifts from Dr. H. Tokuda (University of Tokyo). For detection of cytochrome bo, bd, and F$_{o}$F$_{1}$-ATPase, the proteins were transferred to nitrocellulose membrane. Anti-bd antiserum was a generous gift from Dr. H. Matsuzawa (University of Tokyo). Anti-F$_{o}$F$_{1}$-ATPase antiserum was a generous gift from Dr. M. Futai (Institute of Scientific Industrial Research, Osaka University).

**H$^+$ Pumping Activity in Provacuoles—**Measurement of H$^+$ pumping activity was carried out by the quinacrine fluorescence quenching method (6). Provacuoles (20 μg of protein) were added to 2 ml of the assay buffer (10 mM Tris-HCl, pH 7.4, 2 mM MgCl$_2$, 20 mM KC1, and 30 mM sucrose) containing 1 μM quinacrine hydrochloride. After preincubation for 5 min at 25 °C, NADH or ATP was added. After fluorescence quenching had occurred, KCN or DCCD was added as an inhibitor of the respiratory chain or F$_{o}$F$_{1}$-ATPase.

**Electrical Recording in Provacuoles—**Giant protoplasts were harvested by centrifugation at 740 × g and gently suspended in a small volume of the same medium as that used for cell growth. The giant protoplasts were put on a glass chamber and washed with GPW buffer (10 mM Tris-HCl, pH 7.4, 10 mM MgCl$_2$, and 200 mM sucrose). The GPW buffer was replaced with the Burst buffer containing 210 units/ml of DNase I. After a few minutes, the giant protovacuoles were washed again with the Burst buffer. The chamber was filled carefully with Burst buffer. The patch pipettes (Drummond Scientific Co.) were pulled to a diameter with a resistance of 12.5–25 MΩ (when measured in Burst buffer) using a puller machine (model PC 10, Narishige) and then heat-polished (model MF-90, Narishige). The electrode was gently touched to a giant vacuole with a ZAP pipette technique at 23 °C (1).

**RESULTS**

**Vacuole-like Structures Surrounded by a Single Membrane—**An electron micrograph of a giant protoplast and a vacuole-like structure formed in the protoplast are shown in Fig. 1A. The diameter of the giant protoplast in the figure is about 13 μm and that of the vacuole-like structure is about 10 μm. The original E. coli cell (1 × 2 μm) is shown in Fig. 1B. The membranes of the giant protoplast (indicated by an arrowhead) and that of the giant vacuole-like structure (indicated by an arrow) are shown in Fig. 1C. Compared with membranes of the original cell shown in Fig. 1D, which consist of two membrane structures (two arrows indicating outer membrane and inner membrane, respectively), both of the membranes of the giant protoplast and the giant vacuole-like structure consist of single membrane (Fig. 1C). We took many electron micrographs of the giant protoplasts and the giant vacuole-like structures and obtained the same results. Thus, we conclude that both the giant protoplasts and the giant vacuole-like structures are surrounded by single membrane. It should be noted that most giant protoplasts contained several vacuole-like structures in one protoplast, as will be shown below. No ribosome-like structures are present inside the giant vacuole-like structures (Fig. 1, A and C). We stained the giant protoplasts and the giant vacuole-like structures with DAPI, which stacks between DNA double strands, and investigated whether DNA exists in the vacuole-like structures (Fig. 2). Our results clearly indicate that there is no detectable DNA in the vacuole-like structures (Fig. 2C). However, DNA is present in the cytoplasm of the giant cells (Fig. 2C). Because the vacuole-like structures do not contain ribosomes, DNA, and any other electron-dense materials, we henceforth refer to the vacuole-like structures as “provacuoles.”

Microscopic observations revealed that the giant protoplasts

**FIG. 1.** Electron micrographs of giant protoplast and intact cells of E. coli K002. A, a giant protoplast with a giant vacuole-like structure. B, an intact cell. C, magnification in rectangular frame of A, D, magnification in rectangular frame of B. Scale bars indicate 1 μm (in A and B) or 100 nm (in D). The magnification in C is same as that in D.
of the provacuoles by immunoblot analysis (Western blotting). SecY is a major component of the Sec complex (18, 19). We detected two protein bands that reacted with anti-SecY antibody in both the protoplasts and the provacuolar membranes (Fig. 4A, lanes 1 and 2). The band corresponding to the SecY in the protoplasts was very faint, suggesting that the relative amount of SecY in the protoplasts is small compared with total proteins in the protoplasts. On the other hand, the SecY was clearly detected in the provacuolar membranes, suggesting that the relative amount of SecY compared with other membrane proteins in the provacuolar membranes is enough. The band with lower molecular weight shown in the figure (Fig. 4A, lanes 1 and 2) is a nonspecific one. The α and β subunits of the F_{0}F_{1}-ATPase complex, the major subunits of the complex, were present in the provacuolar membranes prepared from E. coli strain K002, a wild type cell with respect to the F_{0}F_{1}-ATPase (Fig. 4B, lane 1). On the other hand, only faint bands corresponding to the α and β subunits were detected with the provacuolar membranes from strain K003, an F_{0}F_{1}-ATPase negative mutant (Fig. 4B, lane 2). We detected both subunit 1 of the cytochrome bd complex (Fig. 4C) and subunit 1 of the cytochrome bo complex (Fig. 4D) from the terminal oxidase of the respiratory chain in the provacuolar membranes. The amount of the bd and the bo in E. coli cells is greatly affected by the growth phases (20). The bo complex is prominent at the early exponential phase of growth. The bd complex is prominent at late exponential phase. Under the growth conditions in our experiments, the bd was the major terminal oxidase. The amount of bd in the provacuolar membranes was roughly 10 times larger than that of bo in K002. We also checked the amount of bd and bo in the provacuolar membranes from K003, a F_{0}F_{1}-ATPase negative mutant. Again, bd was present in the provacuolar membranes in larger amounts than bo. However, the amount of bo was larger than that in the provacuolar...
membranes of K002 (Fig. 4D). Thus, all of the major transporters in the cytoplasmic membranes tested were present in the provacuolar membranes. Therefore, we conclude that the provacuolar membranes are very similar to the cytoplasmic membranes of intact cells.

**Orientation of the Provacuolar Membranes**—We tested the activity and direction of protein transport via the Sec system in the provacuolar membranes. MBP is a component of the maltose transport system and is located in the periplasmic space of intact E. coli cells. This protein is synthesized by ribosomes in the cytoplasm and excreted to the periplasm through the Sec secretion machinery. A signal peptide at the NH2 terminus of MBP is necessary for the secretion to occur. We constructed a plasmid encoding a fusion protein between MBP and GFP. The GFP portion was attached to the COOH terminus of the MBP. Thus, location of the fused MBP in the cells could easily be detected because of the green fluorescence emitted by GFP. We constructed two types of plasmids that should produce two types of fusion proteins, one possessing no signal peptide (pMAL-cCm-GFP) and the other possessing the signal peptide (pMAL-pCm-GFP). The fusion protein with no signal peptide was detected in the cytoplasm of the giant protoplasts (Fig. 5, A and C). MBP could not be excreted from the cytoplasm without the signal. When the signal peptide is present, the fused MBP was detected mainly inside the provacuoles of the giant protoplasts (Fig. 5, B and D). Faint fluorescent signals were detected in the cytoplasm. This means that the Sec system is functional in the membranes of the provacuoles and that the direction of the secretion (protein transport) is from cytoplasm to the interior of the provacuoles. This indicates that the provacuolar membranes have an everted orientation compared with the cytoplasmic membranes.

We then tested whether the MBP in the provacuoles is in the mature (processed) form or immature (unprocessed) form by immunoblot analysis. We used a plasmid pMAL-pCm encoding a fusion protein MBP-LacZa (α fragment of β-galactosidase).
Expression of the fusion protein from the plasmid gene is under tac promoter. No protein of the giant protoplasts from uninduced cells reacted with antibody against the MBP (Fig. 6, lane 1). Contrary to what was expected, we detected four protein bands that reacted with the antibody in the giant protoplasts prepared from cells induced with IPTG (a gratuitous inducer of the tac promoter) (Fig. 6, lane 2). Among the four, the first two protein bands (top of the gel) corresponded to proteins that reacted with antiserum against LacZ (data not shown). The fourth protein band (the lowest molecular weight) corresponded to the mature MBP (Fig. 6, lane 8). Two dense protein bands (the second and the fourth) were observed in culture medium of the giant protoplasts (Fig. 6, lane 3). Thus, we believe that the first and second bands (in lane 2) correspond to premature and mature MBP-LacZ, respectively. On the other hand, we believe the third and fourth bands (in lane 2) correspond to premature and mature MBP, respectively, both of which were derived from the fusion protein. Mature MBP-LacZ and mature MBP are present in the culture medium. Mature MBP is present in the provacuoles. The absence of mature MBP-LacZ in the provacuoles suggests that the LacZa portion of the fusion protein was cleaved by some unidentified protease. In any case, it is clear that protein-processing secretion machinery is present in the provacuolar membranes.

We confirmed that the provacuoles are everted (orientation is inside-out compared with the cytoplasmic membranes) by measuring direction of H+ transport because of respiration or ATP hydrolysis by the F,F1-ATPase in the isolated provacuoles. We observed quinacrine fluorescence quenching because of respiration (Fig. 7A) or ATP hydrolysis (Fig. 7C), which represents inward H+ transport with the provacuoles prepared from the wild type strain (K002). The NADH-driven H+ movement and the ATP-driven H+ movement were inhibited by KCN, an inhibitor of the respiratory chain, and DCCD, an inhibitor of the F,F1-ATPase, respectively. The fluorescence quenching because of respiration was stronger with the provacuoles from FoF1-ATPase negative mutant K003 than the quenching with provacuoles from wild type K002 (Fig. 7B). The quenching was not detected with the provacuoles from K003 when ATP was added (Fig. 7D). These results support the idea that the provacuoles are everted. No detectable fluorescence quenching caused by addition of NADH or ATP was observed with the giant protoplasts (data not shown).

**Patch Clamp Measurement of Current Because of Respiratory Chain or F,F1-ATPase**—We were able to obtain giant provacuoles possessing activities of the respiratory chain and the F,F1-ATPase. Substrates for the respiratory chain, NADH, and for the F,F1-ATPase, ATP, are accessible to the enzymes responsible for the reactions from exterior of the provacuoles. Thus, it seemed possible to measure current because of H+ transport by the respiratory chain and F,F1-ATPase in the provacuoles by the whole cell recording mode of the patch clamp method. In fact, we detected an inward current larger than 10 pA when NADH (0.25 mM) was added to the assay mixture (Fig. 8A). This current disappeared after NADH was removed from the system. Also we detected a similar current when ATP (1 mM) was introduced; the current disappeared after ATP was removed (Fig. 8B). The ATP-induced current was sensitive to an F,F1-ATPase inhibitor DCCD (30 μM) (Fig. 8C). On the other hand, the NADH-induced current was not sensitive to DCCD (Fig. 8D) but was sensitive to KCN (10 mM) (Fig. 8E). Thus, we have succeeded in measuring current because of H+ transport via the respiratory chain or the F,F1-ATPase in isolated provacuoles of E. coli. These experiments were repeated several times, and very similar results were obtained.

**DISCUSSION**

Two methods are available for direct measurement of current because of ion transport across membranes: the planar lipid bilayer method and the patch clamp method. The former measures current because of an ion-transporting protein in reconstituted lipid bilayers. Purified protein, partially purified protein, or membrane fragments could be used for this method. If purified protein is available, this method is very valuable for measurement of ion transport and characterization of the protein. Hirata et al. (21) and Muneyuki et al. (22) measured an ATP-induced current from F,F1-ATPase in recombinant lipid bilayers, estimated the H+/ATP stoichiometry, and analyzed the basic process of the reaction. However, problems exist in this method with the efficiency of protein incorporation into lipid bilayers and with the orientation of the proteins. The patch clamp method, on the other hand, requires neither purified protein nor reconstitution. Only one cell or membrane vesicle that is large enough for microelectrode pipette is necessary. This method is especially powerful because the function of the target ion transporting system is measurable in native membranes. It is inherently difficult to analyze the function of multicomponent systems that cannot easily be purified as whole complexes, such as the respiratory chain, using the planar lipid bilayer method (23, 24). However, the patch clamp method could be applicable even for these complicated systems. Because so many bacterial mutants are available, the patch clamp method could be extremely useful for the analyses of ion transport systems of bacterial cells. The only problem with using bacteria for application of the patch clamp method is their small
size. We have developed methods to prepare giant protoplasts that contain giant provacuoles from E. coli cells and to isolate the provacuoles from the protoplasts. The provacuoles proved to be very useful for patch clamp analysis. We have succeeded in measuring H⁺ transport via the respiratory chain and the F₀F₁-ATPase in giant protoplasts of E. coli as an electric current using the provacuoles.

It seems that this method is applicable to the analyses of many other ion transport systems of E. coli such as ion transporters or ion-coupled solute transporters and of other bacteria. Kusaka found that giant protoplasts appeared when spheroplasts of B. megaterium were incubated in the presence of an inhibitor of peptidoglycan synthesis and that contents of DNA and RNA in the protoplasts enormously increased (9). A similar phenomenon was observed with E. coli. DNA of control E. coli cells and of giant protoplasts was stained with DAPI. Intensity of the DAPI fluorescence in the giant protoplasts was similar to that in the control cells (data not shown), indicating that much more DNA is present in a giant protoplast compared with a control cell. Synthesis of most of the cellular constituents including DNA, RNA, membrane proteins, and membrane lipids (except cell wall components) is intact in the enlarged protoplasts. These results support the idea that the ceasing of peptidoglycan synthesis interferes with concomitant occurrence between DNA replication and cell division. It seemed likely that giant protoplasts from many bacteria could be formed by similar methods as described in this paper. In fact, we have prepared giant protoplasts and giant provacuoles from several other bacteria. Measurements of ion transport in such giant provacuoles from several bacterial sources are now in progress.

What is (are) the role(s) of the giant provacuoles? In giant cells or protoplasts, if additional membranes are not present, the cells or protoplasts will suffer from shortage of energy or materials produced by membranes, because the ratio of membrane to cell volume becomes very small. During enlargement of the protoplasts, additional membrane structures, provacuoles, were formed. The larger the protoplasts, the more numerous and the larger the provacuoles appeared. Membranes of the provacuoles showed the ability to transduce energy. Activity of H⁺ transport in the provacuoles was fairly high. The estimated turn over rate of H⁺ transport via the F₀F₁-ATPase in the provacuolar membranes was about 600/s, calculating from the current (0.1 pA/μm²) and the amount of enzyme (25). This value is comparable with that (480/s) of ATP hydrolysis by F₁-ATPase (26). Based on the fact that an active respiratory chain and F₀F₁-ATPase exist in the provacuolar membrane, we believe that oxidative phosphorylation takes place in the provacuolar membranes. We observed an increase in the amount of cytochrome bo, which possesses vectorial H⁺ pump activity (27) in provacuoles from mutant K003 (F₀F₁-ATPase negative) compared with those from the wild type K002 (Fig. 4). It looks as if the absence of the F₀F₁-ATPase is compensated for by the increase in the cytochrome bo.

Because the provacuolar membranes have an everted orientation, the interior of the provacuoles corresponds to the periplasmic space of intact cells. There are many proteins in the periplasmic space including binding proteins for solute transport, endoproteases such as DegP (28) and Tsp (29) that hydrolyze proteins damaged by heat shock and so on, and exoprotease, which cleaves degraded peptides to amino acids (30). The amino acids thus produced will be transported to the cytoplasm. Our results suggest the presence of peptidase in the provacuoles (Fig. 6). Vacuoles of Saccharomyces cerevisiae are organelles that have the ability to degrade and recycle unnecessary macromolecular constituents (31, 32). For example, addition of glucose to a yeast culture growing on acetate as a carbon source results in rapid inactivation of gluconeogenic enzymes followed by proteolytic degradation of the inactivated enzymes. The degradation of fructose 1,6-bisphosphatase, a gluconeogenic enzyme, depends on PrA, which is an endoprotease found in vacuoles (33). It is known that vacuoles possess two endoproteases, PrA and PrB, and five exoproteases, ApY, ApI, CpY, CpI, and DAPB-B (32).

The peptidoglycan layer and the outer membrane are present outside of the periplasm in cells. No such structure is present inside of the provacuolar membranes. It is not yet clear whether peptidoglycan is synthesized in the vacuoles in the presence of penicillin, which is necessary for the formation of the giant protoplasts under our conditions, or because of some other unknown reasons. Also it is not clear whether the membrane corresponding to the outer membrane is constructed in the provacuoles because of the absence of the peptidoglycan or for some other unknown reasons. The absence of both the peptidoglycan layer and the outer membrane-like membranes in the provacuoles makes the provacuoles an ideal source of cytoplasmic membranes with an everted orientation.

The provacuoles could be regarded as a reservoir for membrane constituents. Too many cellular constituents will be synthesized in the giant protoplasts. Perhaps the rate of mem-

![Fig. 8. Electrical recording in a provacuole.](http://www.jbc.org/Downloaded from http://www.jbc.org/ by guest on July 25, 2018)
brane lipid synthesis and the rate of membrane protein synthesis are well balanced in the protoplasts. Unbalanced overproduction of membrane proteins would result in formation of inclusion bodies. It is likely that concomitant overproduction of both membrane lipids and membrane proteins results in the formation of intracellular membrane structures such as provacuoles.

The provacuoles may be a sort of super organelle that is formed under certain conditions unfavorable for cells. The fact that even prokaryotic cells have the potential ability to form vacuoles may provide a clue to the origin of vacuoles in eukaryotes such as S. cerevisiae (34, 35).

E. coli is the best characterized microorganism. Many ion transport systems are known in E. coli. Extensive biochemical and genetical analyses of such systems, including gene cloning and sequencing have been done. However, electrophysiological analysis has never been done with E. coli cells except for the investigation on the mechanochemical channel by Kung and co-workers (2). The methods described in this paper have opened a new field of electrophysiology in E. coli cells. Although we mainly used an E. coli mutant K002 that lacks Lpp for the preparation of giant protoplasts and vacuoles, C600 cells, a wild type strain with respect to the Lpp, were also successfully prepared for both the giant protoplasts and vacuoles, C600 cells. Addition of a pertinent drug to the medium for giant protoplast preparation can solve this problem. Thus, structure-function relationship could be investigated by the patch clamp method with the provacuoles derived from cells harboring mutant-type gene. These methods are also applicable to some, perhaps many, other bacteria.

Furthermore, it is possible to inject the giant protoplasts or giant provacuoles with effectors such as inhibitors, stimulators, modifiers, or even antibodies. We can observe their effects directly. Of course, we can inject plasmid DNA into the giant protoplasts. Thus, it is possible to use the giant protoplasts or giant provacuoles as biologically active test tubes or to use E. coli as a living test tube. Biochemically and genetically, E. coli is the best characterized organism. The DNA sequence of the whole genome of this organism has been determined. We believe that the development and establishment of the methods described in this paper are invaluable for studies on ion transport systems and ion channels in E. coli and other bacteria. New ion channels may be discovered in bacterial cells.

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