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Regulation of Cell Volume in *Escherichia coli*: L-form NC7

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The ATPase inhibitor dicyclohexylcarbodiimide (DCCD), which collapse the chemical and electrical components of the proton motive force, caused rapid cell swelling. Addition of extracellular osmotic salt such as $CaCl_2$ prevented the swelling of DCCD-treated cells incubated in NaCl solution. Ionophore nigericin, which collapse the proton gradient by promoting electroneutral K^+/H^+ exchange, and protonophore carbonylcyanide-*m*-chlorophenylhydrazone (CCCP, 10 μM) induced osmotic swelling in NaCl solution, but not in $CaCl_2$. External solutes such as $CaCl_2$ prevented the swelliong of DCCD-treated cells incubated in NaCl. Ionophore valinomycin, collapse membrane potential in the presence of KCl, falled to induce swelling in NaCl solution. These results show that in L-form NC7 the swelling was induced by the collapse of the electrochemical gradient of proton. Therefore, it was postulated that Na^+ that diffused into the cell was extruded by a Na^+/H^+ antiport energized by the proton motive force to maintain constant cell volume.

Introduction

Most bacteria possess rigid peptidoglycan-based cell wall that gives osmotic protection against swelling. However, without a rigid cell walls, organisms such as Mycoplasma rely for the regulation of their cell volume on mechanisms to extrude solutes that enter the cell (Linker and Wilson, 1985; Rottem, *et al.*, 1981). Swelling of Mycoplasma cells in hypotonic solution of NaCl is due to the inward diffusion of NaCl and water as a result of colloid-osmotic and Gibbs-Donnan force caused by intracellular nondiffusable macromolecules (Macknight and Leaf, 1977). It is well-known that many bacterial L-forms isolated from various bacteria species lack rigid peptidoglycan-based cell wall and are osmotically fragile, requiring osmotic stabilizer such as NaCl or KCl (Madrof, 1986). L-form NC7 strain isolated from *E. coli* (Onoda, 1986) also lack rigid cell wall and when the cells are incubated in an hypotonic solution, they swell. It has been observed in L-form NC7 that when cells are incubated in an isoosmotic solution of NaCl, the cells slowly swell. Swelling was prevented by replacing NaCl with $CaCl_2$ as osmotic stabilizer. It has been suggested that swelling was induced by increase in osmotically active Na^+ entering into cell and that regulation of cell volume depends on extrusion of Na^+ from cells.

E. coli has three distinct antiport systems which function in the extrusion of cations from the cytosol, *i.e.* Na^+/proton, K^+/proton and Ca^{2+}/proton antiport systems (Brey

et al., 1978; Brey *et al.*, 1980; Rosen, 1987; Tsuchiya and Takeda, 1979). Three distinct cation/proton antiport systems: K^+/proton, Na^+/proton and Ca^{2+}/proton antiporters were found on everted membrane vesicles of L-form NC7 (Onoda *et al.*, 1989). L-form NC7 possess a proton-translocating F_1F_0-ATPase (Nakano and Onoda, 1988). It is proposed that L-form NC7 regulates its intracellular volume by extrusion of Na^+ through the combined operation of a dicyclohexylcarbodiimide (DCCD)-sensitive H^+-ATPase and an Na^+/proton antiporter. In this paper, the possible roles of H^+ and Na^+ gradients in the regulation of cell volume in this organism are discussed.

Materials and Methods

Organisms and growth conditions

The parent strain, *Escherichia coli* K12 strain 3301 and L-form NC7 (Onoda, 1986) were used in this experiment. Both the strains were grown at 32°C without shaking on the following complex media. NaPY medium contained (per liter) 10 g peptone, 5 g of yeast extract, 2 g of glucose and 0.34 M NaCl. CaPY medium was the same as NaPY medium, except that 0.2 M $CaCl_2$ was used instead of 0.34 M NaCl. The pH value was adjusted to 7.2 with NaOH. In some experiments, Tris(hydroxymethyl)aminomethans was used as adjustment of the pH. Cells were harvested in the exponential phase of growth by centrifugation ($4000\times g$, 15 min), washed once with growth medium supplemented with suitable osmotic stabilizer, and inoculated into culture medium. Growth was monitored by measuring optical density at 600 nm (1 cm path length).

Swelling measurements

The swelling of the L-form cells was determined spectro-photometrically. Cells were harvested at the late exponential or stationary phase of growth. Washed cell suspensions were diluted in solutions of 0.4 M NaCl and 0.3 M $CaCl_2$ to a cell density (A_{600} nm) of 0.3 to 0.5. The cell suspensions were then incubated at 32°C, and cell swelling was observed by measuring the absorbance at 600 nm of the cell suspensions. Results were also expressed as the change in the percentage of initial absorbance with time.

Chemicals

The sources of materials used in this work were as follows: Peptone was purchased from Kyokuto Pharmaceutical Industrial Co., Tokyo. Yeast extract powder was obtained from Oriental Yeast Industrial Co., Tokyo. Valinomycin, nigericin, carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), and tetraphenylphosphonium bromide (TPP) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Dicyclohyxylcarbodiimide (DCCD) and sucrose (RNase-free) from Nacalai Tesque. All of the other chemicals were reagent grade and obtained from commercial sources.

Results

Cell swelling of L-form NC7

Changes in the optical density of no growing cell culture, generally reflect changes of cell volume. These two changes are related in a reciprocal manner: an increase in optical density reflects a decrease in cell volume and a decrease in optical density reflects an increase in cell volume. The swelling of the L-form cells was investigated by monitoring changes in the absorbance of cell suspensions incubated in various osmotic salt solutions. Significant swelling was observed when cells were suspended for several hours in 400 mM-NaCl in the presence or absence of glucose (Fig. 1). The rate and extent of swelling was higher in the presence than the absence of glucose. From the osmotic behavior of the L-form in NaCl solution, a possible explanation for the induced swelling is probably due to increased influx of osmotically active Na ions into the cells by added glucose. The tendency to swell in NaCl solution was the greatest with stationary phase cells (data not shown). Cells exposed to 300 mM $CaCl_2$, however, hardly showed swelling during the incubation (Fig. 1). On the other hand, when glycerol was used as osmolyte, a cell swelling followed by lysis occurred immediately. Glycerol was ineffective as osmoprotectant.

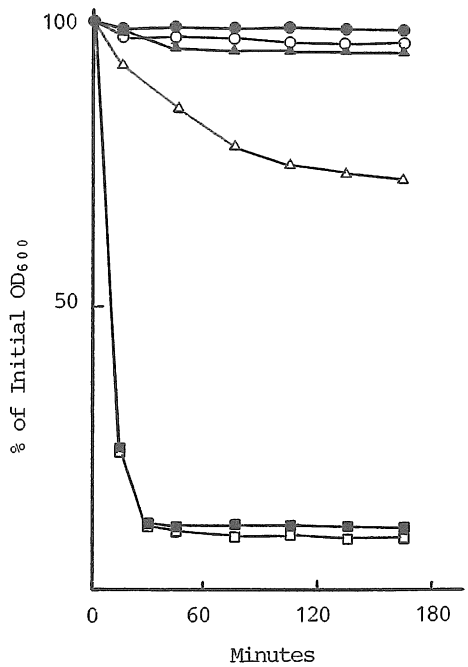

Fig. 1. Swelling of L-form in NaCl, $CaCl_2$, and glycerol solutions. Cells were incubated at 32°C in the presence (open symbols) or absence (solid symbols) of 10 mM glucose in a solution containing 10 mM Tris-HCl buffer (pH 7.6) and 400 mM NaCl (△, ▲), $CaCl_2$ (○, ●) or glycerol (□, ■). Swelling was monitored as described in *Materials and Methods*.

Effect of DCCD on cell swelling

If the ATPase in L-form NC7 strain (Nakano and Onoda, 1988) is involved in

regulation of cell volume, then DCCD, an energy-transfer inhibitor that blocks ATP generation and consumption by binding specifically to ATPase, should stimulate cell swelling and as a result, lead to cell lysis. Fig. 2 shows the effect of DCCD on swelling. Addition of DCCD (20 to 200 μM) in the presence of NaCl caused a very rapid swelling and lysis of the cells. DCCD induced a concentration-dependent swelling of the cells suspended in NaCl solution. At a concentration of 200 μM, DCCD produced a maximal effect on swelling. Various solutes were tested in an attempt to prevent the DCCD-induced swelling and lysis of the cells. Fig. 3 shows effects of different solutes on swelling in the presence or absence of DCCD. Substitution of the NaCl by KCl or choline chloride (data not shown) failed to prevent swelling. The most striking protection was provided by $CaCl_2$ solute (Fig. 3). In the presence of $CaCl_2$, DCCD had no effect on swelling during the first 30 min, and subsequent swelling was strikingly

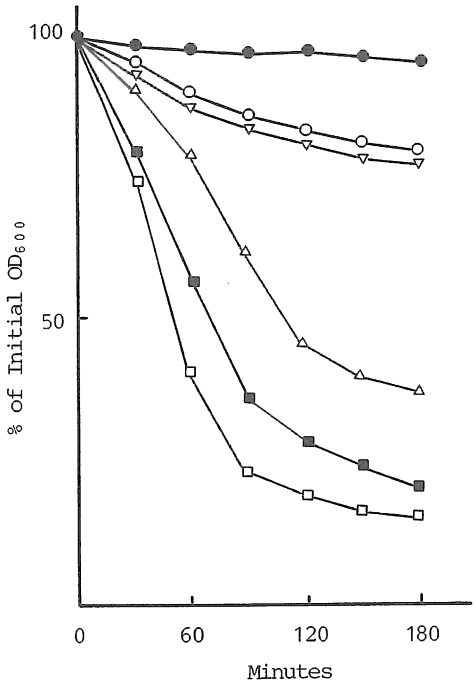

Fig. 2. Effect of DCCD on L-form cell swelling. Washed cells were suspended in 5 ml 400 mM NaCl solutions in 10 mM Tris-HCl (pH 7.6) in the presence (open symbols) or absence (solid symbols) of 10 mM glucose and incubated at 32°C in the following concentrations of DCCD: 0 μM (○, ●), 20 μM (▽), 100 μM (△), 200 μM (□, ■).

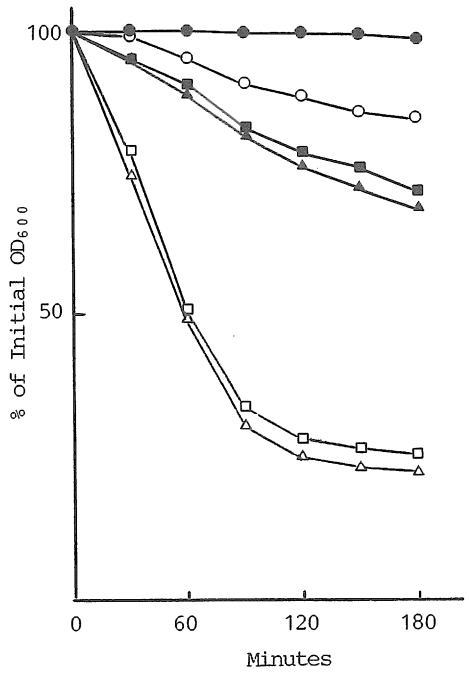

Fig. 3. Effect of DCCD on the swelling in various osmotic salt solutions. Cells were incubated at 32°C in the presence (open symbols) or absence (solid symbols) of 10 mM DCCD in a solution containing 10 mM Tris-HCl (pH 7.6) and the following: 400 mM NaCl (△, ▲), 400 mM KCl (□, ■), or 300 mM $CaCl_2$ (○, ●).

less than that observed on cells suspended in NaCl plus DCCD. Ca^{2+} exhibited a protective effect against swelling even in the presence of DCCD (Fig. 3). In the next experiment, when the cells grown in CaPY medium were suspended in NaCl, KCl, or $CaCl_2$ solutions with or without DCCD, the response to DCCD on swelling of the former cells was very like to that of the cells grown in NaPY medium (Fig. 4). These results suggest that cell swelling is dependent on sorts of external osmotic stabilizer, but not on the precultured conditions of cells. On the other hand, the striking swelling in NaCl or KCl solutions in the presence of DCCD suggests that internal osmotic active Na^+ or K^+, which enter the cells through either specific transport system or non-specific diffusion processes, cause swelling when these ions efflux were blocked by addition of DCCD. Since it is known that DCCD dissipated both the chemical and electrical components of the proton motive force, cell volume regulation aappears to require the presence of an electrochemical potential difference of proton ($\Delta\mu H^+$) across the membrane.

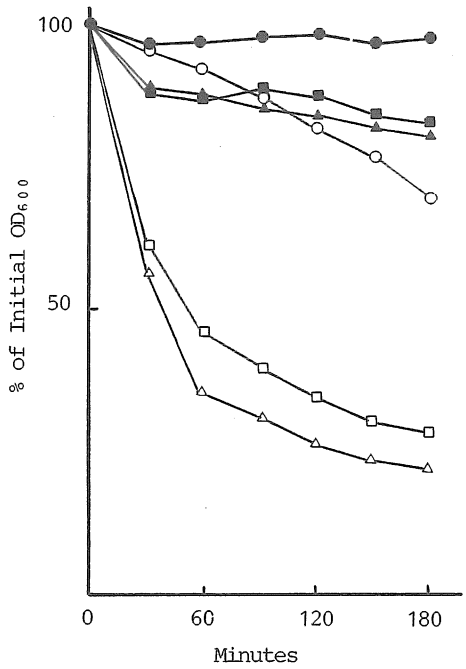

Fig. 4. Effects of DCCD on the swelling of the cells precultured in CaPY medium. The cells were serially grown in CaPY medium and harvested in logarithmic phase. Washed cells were incubated at 32°C in the presence (open symbols) or absence (solid symbols) of 200 μM DCCD in a solution containing 10 mM glucose and 10 mM Tris-HCl (pH 7.6) and the following: 400 mM NaCl (□, ■), 400 mM KCl (△, ▲), or 300 mM $CaCl_2$ (○, ●).

Effect of ionophores on swelling

Several ionophores were used to selectively dissipate either the chemical or electrical components of the proton motive force. If Na ions were extruded by a secondary Na^+/H^+ antiport mechanism, Na^+ efflux should be driven by the proton motive force and inhibited by uncouplers which collapse $\Delta\mu H^+$. The proton ionophore, CCCP,

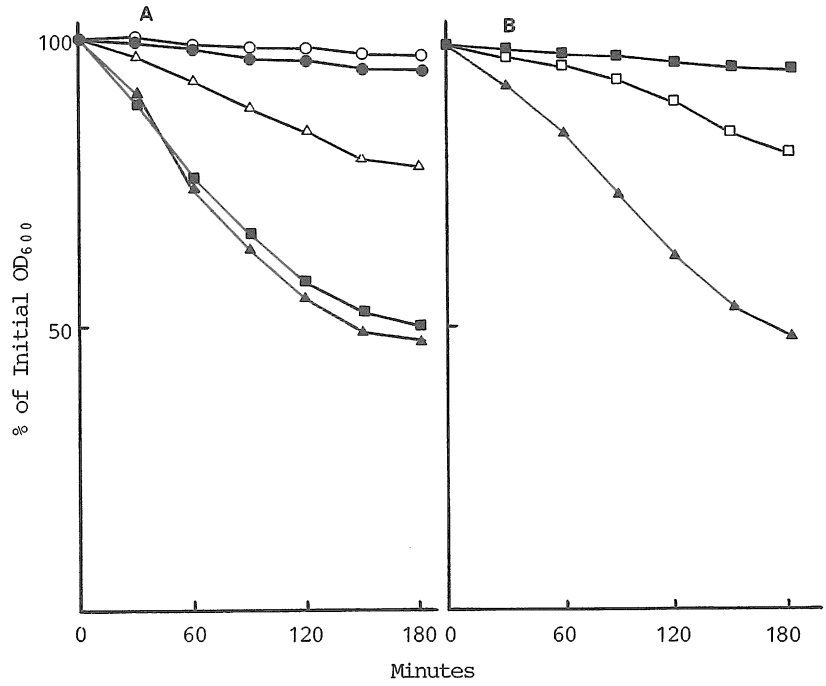

Fig. 5. Effect of CCCP on the swelling. Cells were incubated at 32°C in the following: (A), 400 mM NaCl (△, ▲) or 300 mM $CaCl_2$ (○, ●) solutions containing 10 mM Tris-HCl (pH 7.6) in the presence of glucose. With (▲, ●) or without (△, ○) 20 μM CCCP: ■, 400 mM NaCl solution containing 10 mM Tris-HCl (pH 8.2) with 20 μM CCCP in the presence of glucose: (B), 400 mM plus 0 mM $CaCl_2$ (▲), 400 mM plus 100 mM $CaCl_2$ (□), or 400 mM NaCl plus 200 mM $CaCl_2$ (■), with 20 μM CCCP.

collapse only the chemical components of the proton motive force in alkaline medium (Avetisyan, *et al.*, 1989). The swelling of the cells suspended in alkaline NaCl solution was significantly affected by the addition of CCCP (10 μM) (Fig. 5A). Similar results were obtained with cells suspended in KCl solutions (data not shown). However, the swelling of the cells suspended in 300 mM $CaCl_2$ solution was not affected by the addition of CCCP. In addition, effects of Ca^{2+} on swelling were measured in the combination with NaCl and $CaCl_2$ in the presence of CCCP (Fig. 5B). Furthermore, when CCCP were added to growing media (NaPY and CaPY) at the early exponential phase of growth, 10 μM CCCP completely blocked the cell growth (Fig. 6). These results indicate that CCCP is effective in growing medium containing 0.2 M $CaCl_2$ as well as NaCl. On the other hand, K^+ ionophore valinomycin, which collapse the electrical potential in the presence of external pottasium, induced very little swelling in NaCl or $CaCl_2$ solution with 10 mM KCl (Fig. 7). Tetraphenylphosphonium (TPP), which collapse the membrane potential, acted in analogous fashion to valinomycin.

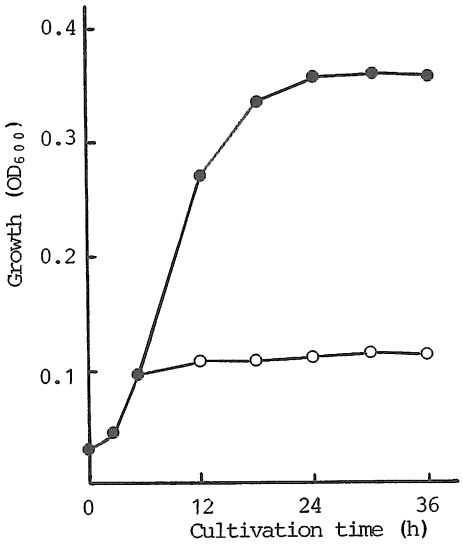

Fig. 6. Effect of CCCP on growth of L-form in CaPY medium. The cells were grown in CaPY medium (pH 7.2) containing 10 mM glucose without shaking at 32°C. ○, 10 μM CCCP added at the point indicated by the arrow; ●, control culture.

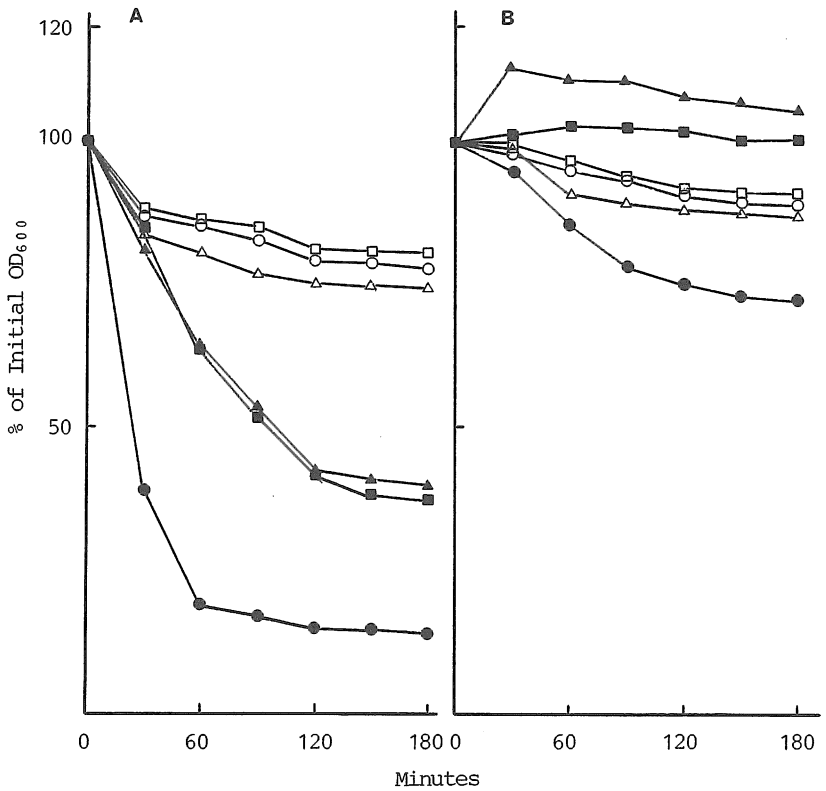

Fig. 7. Effect of ionophores on the swelling. Cells were incubated at 32°C in 400 mM NaCl (A) or 300 mM $CaCl_2$ (B) solutions containing 10 mM Tris-HCl (pH 7.6) in the presence of glucose, and the following: 200 μM DCCD (●); 10 μM nigericin (■); 10 μM nigericin plus 10 μM valinomycin (▲); 10 μM valinomycin (△); 10 μM TPP (□), control (○).

Nigericin as a K^+/H^+ exchanger collapsed the pH gradient in the presence of potassium. Nigericin (plus 10 mM KCl) induced rapid swelling of cells incubated in NaCl solution, but no $CaCl_2$. It would be suggested that the presence of pH gradient can provided the energy to maintain a cell volume.

Discussion

Bacterial L-forms lack a rigid cell wall, are bound by a single membrane, and generally susceptible to osmotic lysis (Madroff, 1986). When L-form cells are incubated in an isoosmotic NaCl solution of high ionic strength, they swell. Swelling was suggested to be due to the inward diffusion of Na^+ and Cl^- into the cell as a result of Gibbs-Donnan forces (Rottem, *et al.*, 1982). It, therefore, seems that volume regulation reflects a balance between active cation and passive anion movements across the membrane. *E. coli,* including L-form NC7, possess a membrane-bound proton pumps, ATPase, suggesting an important role in cell function. We found that the L-form possess a ouabain-insensitive H^+-translocating ATPase (Nakano and Onoda, 1988). Membrane-bound proton pumps are generally believed to maintain $\Delta\mu H^+$ in bacterial cells (Padan, *et al.*, 1981; Smirnova, *et al.*, 1990). The proton electrochemical gradient ($\Delta\mu H^+$) is composed of the membrane potential and the pH gradient across the membrane. In the L-form, an electrogenic proton ATPase generates both ΔpH and $\Delta\Psi$. It is postulated that the activation of an H^+-ATPase resulted in a membrane potential, and ΔpH, which provided the driving force for the inward movement of H^+ *via* the secondary transport systems (K^+/H^+ and Na^+/H^+ antiporters). Therefore, it is expected that the L-form extrudes K^+ or Na^+ by a combination of a proton-translocating ATPase and an K^+ or Na^+/H^+ antiporter. A possible mechanism for Na^+ extrusion found in many bacteria is the pumping of protons *via* the H^+-ATPase and extruding Na^+ by means of the Na^+/H^+ exchange carrier. Stimulation of the rate of swelling by 100 μM DCCD appears to be consistent with blockage of the membrane ATPase by the inhibitor. It is known that the protonophore, CCCP collapses both $\Delta\Psi$ and ΔpH in most organisms (Avetisyan, *et al.*, 1989). In this paper, it was found that swelling of the L-form cells was caused by addition of proton ionophore CCCP. The ionophore nigericin catalyzes electroneutral exchange of protons for monovalent cations with a high specificity for K^+. When the L-form cells suspended in K^+-containing solutions were treated with nigericin, swelling and lysis of the cells was induced during the incubation. In addition, amiloride, a known antagonist of Na^+/H^+ antiporter activity in *E. coli* (Mochizuki-Oda and Oosawa, 1985), also caused significant swelling of the cells. In this experiment, however, the swelling of the L-form cells suspended in an isoosmotic NaCl plus 10 mM KCl solution was not affected by the addition of valinomycin, which collapse membrane potential ($\Delta\Psi$) in the presence of KCl. The *E. coli* cells generally maintains the nonequilibrium transmembrane Na^+ distribution ($[Na^+]_{out} > [Na^+]_{in}$) in high Na concentrations and an inwardly directed Na^+ gradient is supported

by the $\Delta\mu H^+$ driven Na^+/H^+ antiport (Krulwich, 1983; Dibrov, 1991). Swelling and lysis was interpreted to be caused by an increase in intracellular osmotic pressure. Since volume is actively regulated in the L-form, the primary extrusion of an ion than H^+ would be expected to play a central role. Swelling of the L-form seems to occur when proton gradient was collapsed by addition of these ionophores. The suspending cells in an isoosmotic solution of permeable cations result in the diffusion of these cations into the cells, increasing cellular ionic strength. Therefore, this increase would subsequently stimulate the sodium transport to extrude Na^+ and reduce the intracellular osmotic pressure. On the other hand, when the L-form cells was suspended in $CaCl_2$ solution, DCCD induced a little swelling. In addition, when CCCP and nigericin (plus 10 mM KCl) were added to the L-form cells suspended in $CaCl_2$ solution, swelling were hardly observed. We found that 10 μM CCCP used in this experiment inhibited completely the growth of the cells suspended in $CaCl_2$ solution. No swelling in $CaCl_2$ solution seems to indicate that Ca^{2+} enter hardly the cells through either specific transport systems or nonspecific diffusion processes. The mechanism which is involved in the volume regulation will require further study.

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