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# $H^+$ Secretion Induced by Hypertonic Stress in the Cellular Slime Mold *Dictyostelium discoideum*

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**We found that *Dictyostelium* amebas shrank about 50% in 10 min after a change in extracellular osmolarity from 70 mosM to 170-370 mosM. Cells shrunk by the addition of 0.1 M mannitol showed a partial recovery of cell volume in 2 h when the culture medium contained salts. A full recovery of cell volume was observed when 0.1 or 0.2 osM NaCl or KCl was added instead of mannitol. No recovery of cell volume was observed after the addition of 0.1 M mannitol or glycine when the extracellular medium did not contain any salt. An elevation in extracellular pH of starved *Dictyostelium* cells, which is probably due to ammonia production, was suppressed by hypertonic stress. This hypertonic stress-induced suppression of the elevation in extracellular pH was observed only when the extracellular medium contained salt. We also found that cells with artificially elevated intracellular pH withstood hypertonic stress much better than control cells. These results suggest that *Dictyostelium* cells under hypertonic conditions secrete protons and take up charged extracellular osmolytes in exchange. The uptake of charged osmolytes induces the elevation of intracellular osmotic strength and the recovery of cell volume. The elevation of intracellular pH, which is presumably induced by the proton secretion, plays a role in protecting cells from lysis.**

**Key words: cell volume, hypertonic stress, intracellular pH, osmotic response, proton secretion.**

Amebas of the cellular slime mold *Dictyostelium discoideum* grow as single cells feeding on bacteria. When the food bacteria are exhausted, *Dictyostelium* amebas aggregate and form a multicellular organism in which two types of cells, spore cells and stalk cells, are differentiated. *Dictyostelium* cells have been used for studies in cell biology and developmental biology because of their simple growth and developmental life cycle (*1*).

Extracellular osmotic conditions are physiologically important in all organisms (*2*). Hypertonic stress induces drastic change in cell shape. *Dictyostelium* cells, which show active amoeboid movement under normal conditions, lose pseudopods and become round under hypertonic conditions. The structure of the cytoskeleton must be modified and rearranged under hypertonic conditions. Furthermore, the rearrangement of cytoskeletal proteins is important for the protection of cells from lysis under hypertonic conditions. Oyama (*3*) has reported that hypertonic stress induced cGMP accumulation, and Kuwayama *et al.* (*4*) have reported that accumulated cGMP induces myosin phosphorylation and the redistribution of myosin, which are important for survival under hypertonic conditions.

In yeast, a two-component system plays a role in osmotic signal transduction and regulates the MAP kinase cascade (*9, 32*). Although an osmo-sensor and the intracellular signals from it are not well understood in *Dictyostelium*, Schuster *et al.* (*5*) have reported that a two-component histidine kinase (DokA) plays a role in osmotic response in this organism but that the dokA$^-$ mutant can accumulate cGMP in response to hypertonic stress.

Changes in cell volume under hypertonic conditions have not been studied in detail in *Dictyostelium* and osmolytes accumulated under hypertonic conditions have not been identified, although the most striking events under hypertonic conditions are cell shrinkage and the subsequent adaptation (recovery of cell volume). To better understand the responses to hypertonic stress in this organism, we studied the changes in cell volume and mechanisms for the adaptation. We report here that *Dictyostelium* cells pump in extracellular charged osmolytes for the adaptation to hypertonic stress, and that they secrete protons in exchange for the charged osmolytes taken up.

## MATERIALS AND METHODS

*Dictyostelium discoideum* wild-type (NC4) amebas were grown in phosphate buffer with *Escherichia coli* B/r as a food source. Logarithmically growing amebas were washed with chilled distilled water and resuspended in 20 mM phosphate buffer ($K_2HPO_4$-$NaH_2PO_4$, pH 6.4) containing 10 mM KCl (PBK) or the solutions indicated in the figure legends at $1.0-2.0\times10^7$ cells/ml. After the cell suspensions had been shaken at 22°C for 1 h, a half volume of the indicated solutions containing 3×concentrated additives was added. The final concentrations of the additives are

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Abbreviation: PBK, 20 mM phosphate buffer ($K_2HPO_4$-$NaH_2PO_4$, pH 6.4) containing 10 mM KCl.

indicated in the text and figure legends. Details have been previously described (*3*).

To measure cell volume, the cell suspension (0.5 ml) was transferred into a micro-test tube containing 0.5 ml of a chilled solution that was identical to the cell suspension except that it contained 20 mg/ml blue dextran with an average molecular weight of 2,000,000 (Sigma). The mixture in the tube was shaken briefly, then centrifuged at 12,000 rpm for 10 s. The supernatant was removed using an aspirator. The cells and the extracellular liquid were collected at the bottom of the tube by brief centrifugation, then the total weight (test tube plus cell pellet) was measured using a balance (Sartorius R200D). NaOH (0.2 M, 0.75 ml) was added to the tubes and they were stored overnight in a refrigerator. The absorbance at 620 nm ($A_{620}$) was measured using a spectrophotometer (Hitachi U1100) after the tubes had been shaken and centrifuged at 12,000 rpm for 1 min. To measure the background, the cell suspension (0.5 ml) was mixed with 0.5 ml of blue dextran solution, centrifuged and the supernatant was removed. The cell pellet was mixed with fresh chilled solution (1 ml) and centrifuged again. The washed cell pellet was dissolved in NaOH, and $A_{620}$ was measured as described above. The wet cell weight was estimated by subtraction of the dry weight of the tube from the total weight. The actual cell weight was estimated by subtraction from the wet cell weight of the weight of extracellular liquid, which was estimated from the absorbance of blue dextran. We assumed that the specific gravity of the cells was 1.0.

To measure the time course of changes in extracellular pH, the washed cells were suspended in a salt solution containing 15 mM NaCl and 15 mM KCl. The cell suspension (20 ml) was stirred in a 100-ml beaker in a constant temperature room (22°C). The salt solution (10 ml) with or without mannitol (final concentration 0.2 M) was added 1 h after the starvation. A pH electrode was dipped into the cell suspension and pH of the medium was recorded at intervals of 1 min. HCl (final concentration 50 $\mu$M each) was added twice at intervals of 1 min at the end of the experiment.

To test the effects of extracellular substances on changes in extracellular pH, the washed cells suspended in the indicated solutions (1.5 ml each) were cultured with shaking in 20-ml vials for 1 h. Then the indicated osmolyte solutions (0.75 ml) were added. The cell suspensions were sampled at 10 min and the indicated times after the addition and centrifuged at 4,000 rpm for 1 min. The supernatants were transferred to new tubes. The pH of the supernatants in the tubes floating on an ice-water bath was measured with a pH meter (Iwaki pH/ion meter 225).

To test cell lysis by hypertonic stress, the washed cells suspended in 20 mM sodium phosphate buffer (pH 6.0 or 7.0) with or without 10 mM $(NH_4)_2SO_4$ were cultured with shaking at $2\times10^6$ cells/ml for 1 h, then glucose solution (final concentration 0.6 M) was added. The number of surviving cells was counted with a hemocytometer.

All results presented here were reproduced in at least two separate experiments unless specifically noted.

## RESULTS

*Change in Cell Volume under Hypertonic Conditions*— To quantify cell volume, we measured the wet weight of the cell pellets after centrifugation and estimated the amount of the extracellular liquid using blue dextran. The improved method employed here required less than $1\times10^7$ cells/tube and took less than 1 min, while the previous method (*3*) requires 20 times more cells/tube and takes more than 10 min. The estimated cell weight was linearly related to the amount of cells with very small standard deviations (Fig. 1), suggesting that the estimation is accurate.

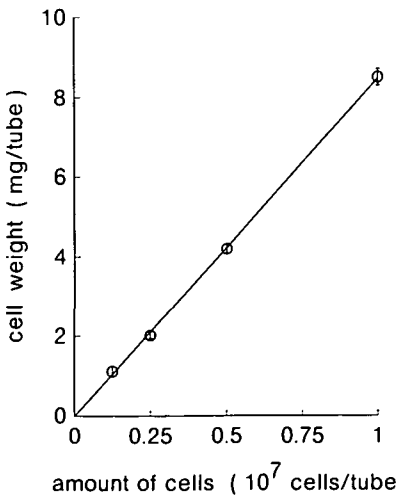


Fig. 1. **Measurement of cell volume using blue dextran.** Vegetative amebas at $0.5\text{-}2.0\times10^7$ cells/ml in PBK were cultured with shaking for 1 h. The cell suspension (0.5 ml) was sampled and mixed with 0.5 ml of blue dextran solution dissolved in PBK and centrifuged. The cell pellet was weighed, then dissolved in 0.75 ml NaOH, and the absorption of blue dextran ($A_{620}$) was measured. The actual cell weight was estimated by subtraction of the weight of extracellular liquid, which was estimated using blue dextran, from the wet weight of the cell pellet. The mean and standard deviation of four determinations are shown.

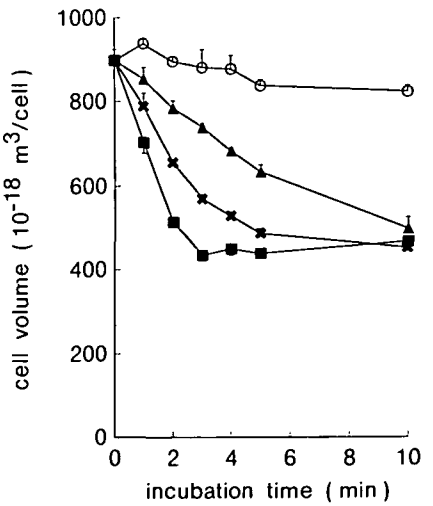


Fig. 2. **Mannitol-induced decrease in cell volume in PBK.** Vegetative amebas were cultured with shaking in PBK (about 0.07 osM) for 1 h, then a half volume of mannitol solution dissolved in PBK was added at 0 time. The final concentration of mannitol was 0 (open circles), 0.1 M (closed triangles), 0.2 M (crosses), or 0.3 M (closed squares). The indicated times show the start of the centrifugation. The mean and standard deviation of three determinations are shown.

The volume of *Dictyostelium* cells starved for 1 h in PBK (*ca.* 70 mosM) was $845 \pm 110 \times 10^{-18}$ m$^3$/cell (nine separate experiments). When mannitol solution (final concentration 0.1 to 0.3 M) dissolved in PBK was added to the cell suspension, cells shrank about 50% within 10 min (Fig. 2). Hypertonic stress of 0.1 osM consistently induced less cell shrinkage than 0.2 or 0.3 osM hypertonic stress, and more severe hypertonic stress induced faster shrinkage (Fig. 2). The shrunk cells gradually recovered during continued culture under 0.1 M mannitol conditions (Fig. 3). Cell volume under 0.1 M mannitol conditions did not reach the control level, although the starved cells in PBK alone gradually decreased in size (Fig. 3). No cell volume increase was observed with 0.2 M mannitol (Fig. 3).

To test whether *Dictyostelium* cells restore their cell volume by the uptake of extracellular osmolytes, the culture medium and the osmolyte were changed. When NaCl or KCl was added instead of mannitol to cells cultured in PBK, cell volume was fully restored even at 0.2 osM (Fig. 4). No specific requirement for sodium ion or potassium ion was observed since cells cultured in 30 mM NaCl or KCl recovered their cell volume after the addition of 0.1 M NaCl or KCl (data not shown). When *Dictyostelium* cells were cultured in 60 mM mannitol instead of PBK, they showed no cell volume increase after the addition of 0.1 M mannitol or glycine, a neutral amino acid (Fig. 5). These results suggest that *Dictyostelium* cells recover their cell volume by the uptake of extracellular charged osmolytes, but that they cannot utilize uncharged extracellular os-

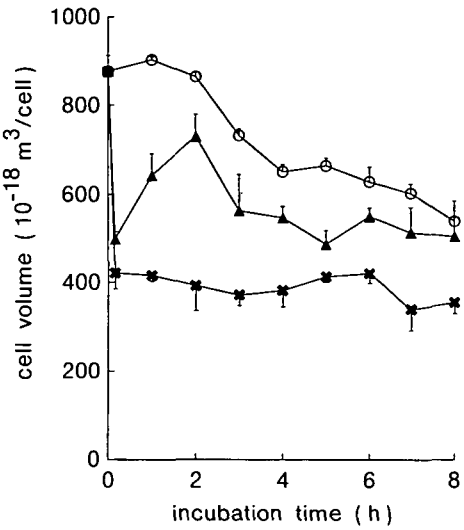


Fig. 3. **Mannitol-induced changes in cell volume in PBK.** Vegetative amebas were cultured with shaking in PBK for 1 h, then a half volume of mannitol solution dissolved in PBK was added at 0 time. The final concentration of mannitol was 0 (open circles), 0.1 M (closed triangles), or 0.2 M (crosses). Culture longer than 4 h was performed only in this experiment. The mean and standard deviation of three determinations are shown.

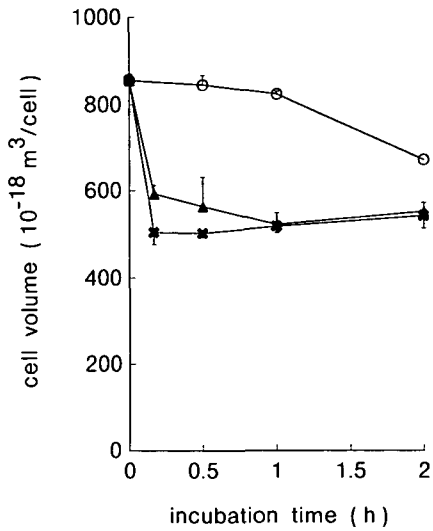


Fig. 5. **Mannitol- or glycine-induced changes in cell volume without extracellular salt.** Vegetative amebas were cultured with shaking in 60 mM mannitol instead of PBK for 1 h, then a half volume of mannitol or glycine solution dissolved in 60 mM mannitol was added at 0 time. The final concentration was 60 mM mannitol (open circles), 0.16 M mannitol (closed triangles), or 0.1 M glycine in 60 mM mannitol (crosses). The mean and standard deviation of three determinations are shown.

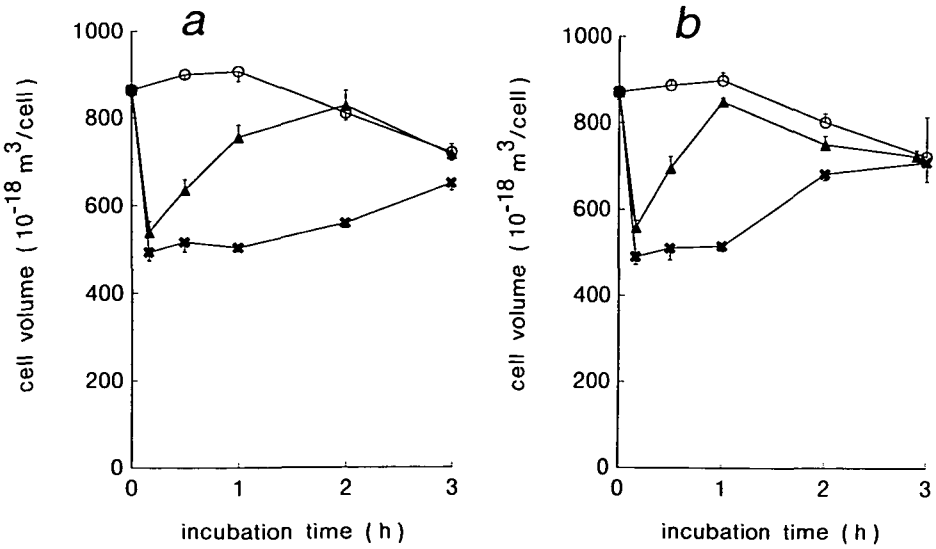


Fig. 4. **NaCl- or KCl-induced changes in cell volume in PBK.** Vegetative amebas were cultured with shaking in PBK for 1 h, then a half volume of NaCl (a) or KCl (b) solution dissolved in PBK was added at 0 time. The final concentration of NaCl or KCl was 0 (open circles), 0.05 M (closed triangles), or 0.1 M (crosses). The mean and standard deviation of three determinations are shown.

molytes such as mannitol and glycine.

Cells under 0.1 osM mannitol, NaCl or KCl conditions resumed pseudopod formation within 1 h, although the recovery of cell volume was partial under 0.1 osM mannitol conditions. Cells under 0.2 osM conditions remained round and showed little pseudopod formation, although cell volume was restored under 0.2 osM NaCl or KCl conditions.

*Proton Secretion Induced by Hypertonic Stress*—Changes in extracellular pH were measured to test whether proton movement plays a role in the uptake of extracellular charged osmolytes under hypertonic conditions (Fig. 6). When a fresh salt solution (15 mM NaCl + 15 mM KCl) was added to cells cultured in the same salt solution, extracellular pH rose gradually after a sharp drop (Fig. 6a), as reported previously (*6, 25*). The sharp drop in extracellular pH was variable among the experiments (data not shown). This drop may be due mainly to $H_2CO_3$ dissolved in the fresh salt solution, since it was also observed in the absence of cells. The gradual elevation is probably due to the production of ammonia by starved *Dictyostelium* cells (*7*).

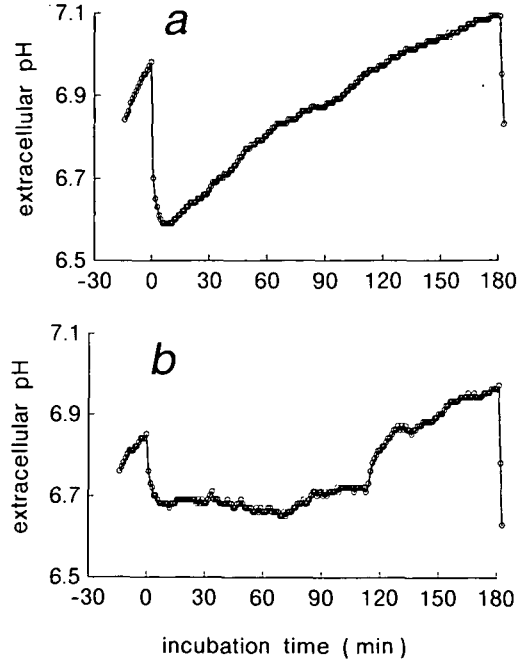


Fig. 6. **Mannitol-induced changes in extracellular pH in 15 mM NaCl and KCl.** Vegetative amebas (20 ml) were cultured with stirring in a salt solution containing 15 mM NaCl and 15 mM KCl for 1 h, then 10 ml of the salt solution without (a) or with (b) mannitol (final concentration 0.2 M) was added at 0 time. Extracellular pH was recorded at intervals of 1 min by use of a pH electrode. HCl (final concentration 50 $\mu$M each) was added twice at intervals of 1 min at the end of the experiments.

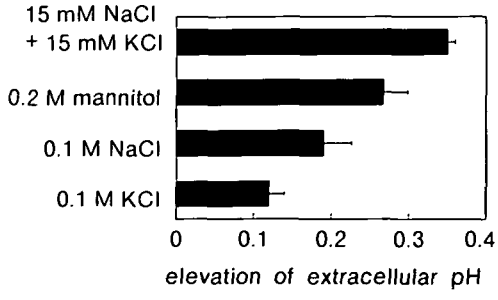


Fig. 7. **Effects of mannitol, NaCl, and KCl on the elevation of extracellular pH.** The washed cells suspended in the salt solution (15 mM NaCl and 15 mM KCl) were cultured with shaking for 1 h, then the indicated osmolyte solutions were added. The cell suspensions were sampled 10 min and 2 h after the addition and centrifuged at 4,000 rpm for 1 min. The supernatants were transferred to new tubes, and their pH was measured with a pH meter. Differences in extracellular pH between 10 min and 2 h are shown as mean and standard deviation of three determinations.

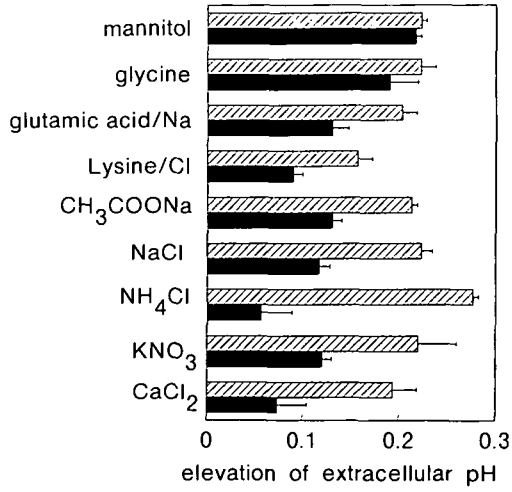


Fig. 8. **Effects of culture media on the mannitol-induced suppression of the elevation of extracellular pH.** The washed cells were cultured with shaking in the indicated solutions (60 mosM) for 1 h, then the indicated solutions (shaded columns) or the indicated solutions containing mannitol (final concentration 0.2 M) (closed columns) were added. The cell suspensions were sampled 10 min and 75 min after the addition and centrifuged at 4,000 rpm for 1 min. The supernatants were transferred to new tubes, and their pH was measured with a pH meter. Differences in extracellular pH between 10 min and 75 min are shown as mean and standard deviation of three determinations.

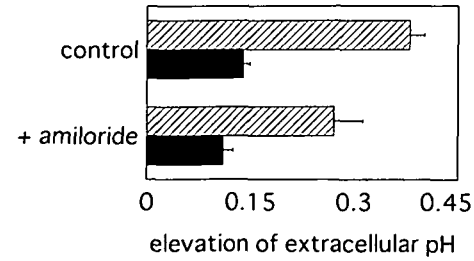


Fig. 9. **Effects of amiloride on the hypertonic stress-induced suppression of the elevation of extracellular pH.** The washed cells were cultured with shaking in 30 mM NaCl with or without 1.5 mM amiloride for 1 h, then NaCl solution was added. The final concentration of amiloride was 1 mM after the addition of NaCl solution, and the final concentration of NaCl was 30 mM (shaded columns) or 130 mM (closed columns). The cell suspensions were sampled 10 min and 75 min after the addition and centrifuged at 4,000 rpm for 1 min. The supernatants were transferred to new tubes, and their pH was measured. Differences in extracellular pH between 10 and 75 min are shown as mean and standard deviation of four determinations.

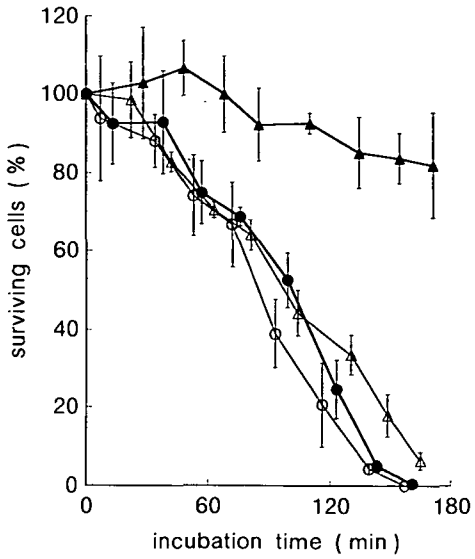


Fig. 10. **Effects of intracellular pH on hypertonic stress-induced cell lysis.** The washed cells suspended in 20 mM sodium phosphate buffer pH 6.0 (circles) or pH 7.0 (triangles) with (closed symbols) or without (open symbols) 10 mM $(NH_4)_2SO_4$ were cultured with shaking for 1 h, then glucose solution (final concentration 0.6 M) was added. The number of surviving cells was counted with a hemocytometer. The mean and standard deviation of four determinations are shown.

The addition of 0.2 M mannitol suppressed the gradual elevation in extracellular pH for 2 h (Fig. 6b). Hypertonic stress not only suppressed the gradual elevation but also repeatedly induced a transient drop in extracellular pH during this period (first 2 h). Thereafter, extracellular pH rose stepwise (Fig. 6b). These transient drops in extracellular pH were small but consistently observed, and this type of fluctuation was not observed in the control experiments (Fig. 6). The addition of HCl at the end of the experiments induced a roughly identical drop in extracellular pH both in the control and in the hypertonic conditions, suggesting that both culture media have a similar buffer action (Fig. 6).

NaCl and KCl suppressed the elevation of extracellular pH for longer than did mannitol, since they induced severer suppression than mannitol at 120 min after the addition (Fig. 7) but roughly the same suppression at 75 min (data not shown). The hypertonic stress-induced suppression was consistently observed when the culture medium contained charged osmolytes, including sodium glutamic acid, lysine hydrochloride, and sodium acetate (Fig. 8), suggesting that this suppression does not depend on a specific anion or cation. Amiloride, a Na/proton pump inhibitor, had little effect on the suppression (Fig. 9). The suppression was not observed when the extracellular medium did not contain a charged osmolyte (Fig. 8). These results suggest that the hypertonic stress-induced suppression of the elevation of extracellular pH is coupled with the recovery of cell volume, and that cells under hypertonic conditions secrete protons and take up charged extracellular osmolytes in exchange.

*Defense against Cell Lysis under Hypertonic Conditions*—The hypertonic stress-induced secretion of protons could cause the elevation of intracellular pH. We monitored cell lysis under hypertonic conditions to test whether the elevation of intracellular pH has any effect on the response to hypertonic stress (Fig. 10). Cells cultured in 20 mM phosphate buffer (pH 6.0 and pH 7.0) lysed within 2 h after the addition of glucose (final concentration 0.6 M). Addition of 10 mM $(NH_4)_2SO_4$ significantly improved the survival rate at pH 7.0, but not at pH 6.0 (Fig. 10).

## DISCUSSION

*Changes in Cell Volume under Hypertonic Conditions*—The volume of *Dictyostelium* cells estimated here ($845 \pm 110 \times 10^{-18}$ m$^3$/cell) is consistent with the previous estimation in which 20 times more cells/tube was used for the assay (*3*). Ashworth and Watts (*8*) and Bonner and Frascella (*31*) have reported that the diameter of *Dictyostelium discoideum* NC4 cells was 11 $\mu$m, suggesting that the cell volume is $697 \times 10^{-18}$ m$^3$/cell. Their estimate is slightly smaller than ours, although Ashworth and Watts (*8*) used swollen cells suspended in distilled water. The reason for this difference is not clear.

*Dictyostelium* cells transferred into 0.1 to 0.3 osM hypertonic conditions shrink about 50%. Hypertonic stress of 0.1 osM consistently induces slightly less shrinkage than 0.2 or 0.3 osM hypertonic stress (Fig. 2), suggesting that the extracellular medium is still hypertonic after the shrinkage under 0.2 and 0.3 osM hypertonic conditions and that the extracellular osmotic strength (about 170 mosM) and the intracellular osmotic strength become isotonic after the shrinkage under 0.1 osM hypertonic conditions. If intracellular osmotic strength is 170 mosM after the 50% cell shrinkage, the intracellular osmotic strength of cells cultured in PBK would be about 85 mosM. This estimate is consistent with the fact that *Dictyostelium* cells cultured in PBK (*ca.* 70 mosM) have contractile vacuoles.

Our results suggest that *Dictyostelium* cells recover their cell volume by taking up charged extracellular osmolytes into their cytoplasm (Fig. 11), but that they are unable to pump in uncharged extracellular osmolytes such as mannitol or glycine (Fig. 11). Our results also suggest that the production of small organic osmolytes such as amino acids and sugars from proteins and polysaccharides does not play a major role in the response to hypertonic stress in this organism. Therefore, although *Dictyostelium* and yeast have a common mechanism of osmotic response in which a two-component histidine kinase plays a role (*5, 9*), *Dictyostelium* cells cope with hypertonic stress differently from yeast cells, which accumulate glycerol under hypertonic conditions (*10*).

*Proton Secretion Induced by Hypertonic Stress*—Hypertonic stress not only suppresses the gradual elevation of extracellular pH but also repeatedly induces a transient drop in extracellular pH (Fig. 6). It has no general inhibitory effect on the production of ammonia and other basic substances, however, since the addition of mannitol without a charged extracellular osmolyte does not suppress the elevation of extracellular pH (Fig. 8). These results suggest that the hypertonic stress-induced suppression of the elevation of extracellular pH is the result of proton secretion rather than inhibition of the production of ammonia and other basic substances (Fig. 11).

The hypertonic stress-induced proton secretion is closely related to the uptake of charged extracellular osmolytes

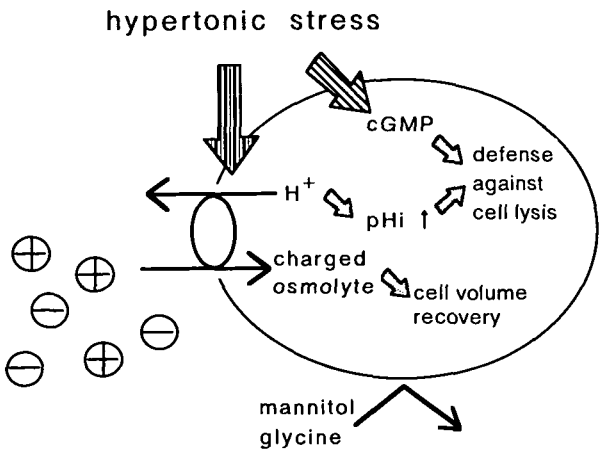


Fig. 11. **Schematic model of the hypertonic stress-induced responses in a *Dictyostelium* cell.** Hypertonic stress induces cGMP accumulation, proton secretion and the uptake of charged extracellular osmolytes. Proton secretion is closely related to the uptake. The uptake of charged extracellular osmolytes induces the recovery in cell volume, and proton secretion induces the elevation of intracellular pH. The alkalization of intracellular pH plays a role in defense against cell lysis in concert with cGMP. *Dictyostelium* cells cannot pump in uncharged extracellular osmolytes such as mannitol and glycine.

(Fig. 11). A Na/proton pump has been reported to play a role in the uptake of extracellular sodium ions and the regulation of intracellular pH in many organisms. However, a Na/proton pump seems not to be specifically required for the adaptation in *Dictyostelium* (*11*), since cell volume recovery (data not shown) and the hypertonic stress-induced proton secretion (Fig. 8) occur without extracellular sodium ion, and the proton secretion is not suppressed by the addition of up to 1 mM amiloride, a Na/proton pump inhibitor (Fig. 9).

*Dictyostelium* cells have two types of proton pumps, a plasma membrane proton pump (*12-14*) and a vacuolar proton pump (*15, 16*). The vacuolar proton pump is located mainly in the contractile system in *Dictyostelium* (*17-19*) and supposedly plays a major role in ion and water transport in that organelle. Nishi and Yagi (*33*) have reported that high salt induces the rapid activation of plasma membrane proton pump in a yeast, *Zygosaccharomyces rouxii*. It would be interesting to determine which proton pump participates in the response to hypertonic stress in *Dictyostelium*.

The changes in extracellular pH under hypertonic conditions, the transient drops during the first 2 h and the stepwise elevation afterwards, seem to occur periodically (Fig. 6). These changes in extracellular pH may reflect oscillatory changes in pH modulators such as proton pumps and sensing mechanisms. Since oscillatory changes are known to occur during the aggregation stage, it is possible that the periodic change observed under hypertonic conditions is related to the autonomous oscillation observed during the aggregation stage.

Hypertonic stress is known to induce cGMP accumulation (Fig. 11) (*3*). Hypertonic stress (0.2 M mannitol) without extracellular salt induced the accumulation of cGMP (data not shown), but the elevation of extracellular pH is not suppressed (Fig. 8). In PBK containing 0.1 M mannitol, however, cell volume recovered (Fig. 3), but little cGMP was accumulated (data not shown). These results suggest that the intracellular signaling pathway inducing proton secretion and salt uptake is at least partly independent of that inducing cGMP accumulation (Fig. 11).

*Alkalization of Intracellular pH and Defense against Cell Lysis under Hypertonic Conditions*—Cells cultured at pH 7 with ammonium sulfate withstand hypertonic stress much better than control cells (Fig. 10). It has been reported that cytoplasmic pH and vacuolar pH are elevated under these conditions (*20, 21*). Since hypertonic stress induces proton secretion, which could induce the elevation of intracellular pH, the alkalization is likely to play a role in the protection of cells from lysis under hypertonic conditions (Fig. 11).

The alkalization of vacuolar pH could modify the activity and/or the function of vacuoles. Since vacuoles play an important role in the transport of salts and water, the alkalization of vacuoles could participate in the osmotic response. Kuwayama *et al.* (*4*) reported that hypertonic stress induces the redistribution of myosin and actin and that the redistribution of myosin is mediated by cGMP. Since pH modifies the activity of some of the regulatory proteins for actin (*22-24, 30*), the hypertonic stress-induced alkalization of the cytoplasm could play a role in the redistribution of cytoskeletal proteins.

A chemoattractant (cAMP) is known to induce the elevation of intracellular pH (*26*) and cGMP accumulation (*27-29*), both of which are thought to participate in the redistribution of cytoskeletal proteins and chemotactic cell movement. Our findings suggest that *Dictyostelium* cells utilize the same mechanism for chemoattractant-induced cell movement and osmotic responses.

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