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Different patterns of cell volume regulation in hyposmotic media between attached and suspended HeLa cells

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Both attached and suspended HeLa cells swelled in a medium of a hypotonic osmolality of 235 mosmol/kg H_2O. When the osmolality was further decreased to 166 mosmol/kg H_2O, attached cells instantly swelled and then rapidly lost water and K^+, followed by slow gains of them. Suspended cells instantly swelled and then K^+ loss and regulatory volume decrease (RVD) occurred. Neither 0.1 mM ouabain nor 10 mM TEA changed the water loss of attached cells, whereas ouabain inhibited RVD of suspended cells. Quinine (1 mM) inhibited water losses from both cells and comparison of the losses implies stronger activation of K^+ channel in attached cells than in suspended cells. Omission of medium Ca^{2+} or addition of 10 mM $BaCl_2$ inhibited RVD in part. These results suggest that hyposmotic stress induces net water loss from attached cells, associated with K^+ release through the Ca^{2+}-dependent K^+ channel. Suspended cells osmotically swell, followed by RVD with K^+ and Na^+ releases through the K^+ channel and Na^+-pump, respectively. The different patterns of volume changes may relate to the difference of activity or time of activation of the K^+ channel between both cells.

Introduction

Cell volumes of many types of cells increase rapidly after incubation in hyposmotic media, followed by their gradual decrease, i.e., regulatory volume decrease (RVD). Among the cells, some are known almost completely to restore their volumes to normal levels. These include human lymphocytes [1,2], mouse lymphoma cells [3], mouse ascites tumor cells [4], opossum renal cells [5], rabbit medullary thick ascending limb cells [6], Chinese hamster ovary cells [7], *Amphiuma* red cells [8] and human intestine 407 cells [9]. RVD occurs also in other types of cells including rabbit red cells [10], but their volumes do not completely return to the normal values. In a few types of cells such as human red cells and rat red cells [11], RVD is not observed after the hyposmotic swelling. The occurrence of RVD has been reported not only for the separated cells but also for rabbit proximal nephron perfused with a hyposmotic solution [12]. Moreover, the presence of high concentrations of highly permeable substances such as urea and KCl in isosmotic media is known to induce RVD of isolated toad bladder cells [13] and HeLa cells [14], respectively. Therefore, RVD seems to be a phenomenon common to different types of cells and probably tissues except a few limited types of cells, but the degree of volume recovery would differ depending on cell types and incubation conditions.

In contrast to the hyposmotic exposures, certain types of cells are known immediately to shrink under hyperosmotic conditions, followed by gradual increase in cell volumes, i.e., regulatory volume increase (RVI). This has been reported for duck red cells [15], *Amphiuma* red cells [8] and rat red cells [11]. In some other cells including mouse ascites tumor cells [16] and opossum renal cells [17], RVI is not found in hyperosmotic media, but the similar process of volume increase occurs upon medium replacement with isosmotic media after an exposure to hyposmotic conditions.

The mechanism of RVD involves water extrusion accompanied with K^+ loss, after cell swelling caused by the difference of osmotic pressure. The K^+ efflux may be carried out through either activated K^+ channels coupled with Cl^- efflux via Cl^- channel or parallel K^+/H^+ and Cl^-/HCO_3^- exchangers. The former system is reported for HeLa cells in high-K^+ media [14] and mouse ascites tumor cells [4], while the latter is

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found in *Amphiuma* red cells [8]. On the other hand, RVI is related to water gain accompanied with Na^+ influx through either activated $Na^+/K^+/Cl^-$-cotransport as in duck red cells [15] or parallel Na^+/H^+ and Cl^-/HCO_3^- exchanges in human lymphocytes [2].

HeLa cells in suspensions have been reported rapidly to swell in hyposmotic media and shrink in hyperosmotic media. Their volume does not change any further in these media for following 30 min [18]. The similar rapid swelling of suspended HeLa cells was observed in hyposmotic media of various osmolalities in the present study, but RVD occurred after the swelling. The difference between the reported and the present results of HeLa cells would be attributed to different incubation conditions. The same cells when attached to culture dishes swelled similarly in a hyposmotic medium of the osmolarity of 235 mosmol/kg H_2O as ideal osmometers, without any succeeding change in the cellular water content. Conversely, the attached cells rapidly lost water, when osmolality of the medium was further reduced to 166 mosmol/kg H_2O or less. This was followed by water gain seemingly like RVI reported for other cells in hyperosmotic solutions.

In the present paper, we showed distinctive behaviors of attached and suspended HeLa cells with respect to volume change in hyposmotic media, and intended to elucidate the mechanisms of membrane water and cation transports supporting the different patterns of volume change.

Materials and Methods

Cell cultures. HeLa S3 cells purchased from Flow Laboratories were serially cultivated in glass culture flasks containing 10 ml of a modified Eagle's minimum essential medium (mMEM) [19] supplemented with 10% (v/v) calf serum. The cells in growing cultures were dispersed with 0.5% trypsin and resuspended with the same culture medium at a density of $5 \cdot 10^4$ cells/ml and inoculated in plastic culture dishes (60 mm diameter, Corning) containing 5 ml of the same culture medium. These dishes were placed at 37°C for another period of 2 days in a CO_2 incubator in humid air containing 5% CO_2.

Cell incubations in hyposmotic media. Isosmotic and hyposmotic media had the same composition of inorganic salts with mMEM, except that the media contained different concentrations of NaCl, and KCl in the absence of amino acids, vitamins and bicarbonate. The concentrations of NaCl and KCl in the isosmotic medium were 145 and 5 mM, while the hyposmotic media consisted of the two cation concentrations of 3/4, 1/2 and 1/4 those of the isosmotic medium. The osmolalities of the isosmotic and the hyposmotic media were 301, 235, 166 and 95 mosmol/kg H_2O, as determined with an osmometer (Osmostat OM-6020, Kyoto Daiichi Kagaku). The cells attached to the culture dishes were once washed with either one of the hyposmotic media and incubated in 5 ml of the same medium. For making cell suspensions, the attached cells were gently washed with Dulbecco's phosphate-buffered saline (PBS) and dispersed by tryptic digestion. The cells were washed once by centrifugation at $12.6 \times g$ for 3 min. They were reincubated with the conditioned medium and allowed to stand for 30 min at 37°C to stabilize the cell membrane [20]. Thereafter, they were again washed with the isosmotic medium by centrifugation, resuspended with the same medium and left for another 30 min at 37°C. Finally, the cells were centrifuged and resuspended in the isosmotic or the hyposmotic medium. All the media used for cell washing and incubations contained 20 mM Hepes (pH 7.2).

To deprive intracellular Ca^{2+}, the attached cells were incubated for 10 min with the Ca^{2+}-free isosmotic medium containing 1 mM EGTA and 10 μM BAPTA-AM. The medium was then replaced with the Ca^{2+}-free hyposmotic medium in the presence of 1 mM EGTA.

Determinations of cell water and cell volume. The water contents of attached cells were determined with [^{14}C]urea (specific activity 1.94 GBq/mmol, Amersham) by the procedures described in a previous paper [14]. The radioactive isotope was diluted to the activity of 2 μCi/ml with the isosmotic or hyposmotic medium before use. Interference of extracellular water was preliminarily tested by double-labeling with 1.5 μCi/ml of [^{3}H]sucrose (specific activity 495.8 GBq/mmol, NEN) and 2 μCi/ml of [^{14}C]urea (see above). Result showed that extracellular water was almost removed by washings and did not significantly interfere with determination of the cellular water content. Only the water contents of suspended cells shown in Fig. 4A were determined by the radioactive urea. Also, there was no significant contamination of extracellular water in this case, as checked by the double-labeling method used together with centrifugation in the presence of silicone oil (see below). The water contents were expressed as μl/mg of cell protein.

To detect quick change in cell volume of the attached cells in a short period within 2 min, microphotographs of the cells were taken serially at an interval of 3–5 s. Cell volume was determined semi-quantitatively by measuring surface areas of the cells and expressed as the ratio of value of $(\text{surface area})^{3/2}$ at any time to that at time zero.

Cell volume of suspended cells was assayed electronically with a new system developed in collaboration with Department of Electric and Electronic Engineering of our university. The system consisted of an electronic cell counter (Sysmex F-300, TOA Medical Electronics) modified and equipped with a constant voltage source, an A/D converter, an interface board and a

personal computer (PC9801UX, NEC) with a software for analyzing cell volume and related parameters. For determining diameters and volumes of the cells, the system was calibrated with polybead-polystyrene microshperes of various mean diameters between 5.55 and 25.7 μm (Polysciences) and pollens of paper mulberry (15.9 μm mean diameter, Polysciences) suspended in PBS. SD of the means were also taken into consideration for the calibration. Mean ± S.D. of diameters of the particles in each group was preliminarily determined by measuring more than 100 particles with a microscopic apparatus (OSM-D2, Olympus Optics) and mean cell volume and its S.D. were calculated. The cell density suitable for the electronic assay was 10^4–10^5/ml. Mean diameters of the cells suspended in the hyposmotic media were also determined by the microscopic method to compare with results obtained by the electronic system. Statistic analysis by Student's t-test showed that diameters measured by the two different methods were not significantly different.

Assays of cations and protein. When Na^+ and K^+ of attached cells were assayed, the culture medium was discarded and cells were washed six times with cold $LiNO_3$ solutions in less than 15 s by the similar procedures described in a previous paper [21], except that various concentrations of the nitrate salt was used in the present study. Because, the osmolalities of $LiNO_3$ must be adjusted to equal those of the incubation media, i.e., 301, 235, 166 and 95 mosmol/kg H_2O. After the washing, the cells were scraped off by a silicon rubber policeman and suspended in 3.5 ml cold double-distilled water. A 2 ml part of the cell suspension was left at least overnight at room temperature to lyze the cells completely and was used for assay of the cations. Another 1 ml aliquot was used for protein assay.

For assaying Na^+ and K^+ of suspended cells, cell densities were increased to about $8 \cdot 10^5$/ml. The suspensions were first chilled in an ice-bath and 1 ml aliquots of the suspensions were overlaid on 1.5 ml of an ice-cold silicone oil (about 3 cm depth) placed in centrifuge tubes. The suitable oil was a 1 : 13 mixture of two different silicone oils (pure grade, Shin Etu), i.e., one was of 0.82 and 1 cps and the other was of 1.02 and 1000 cps in specific gravity and viscosity, respectively. The chilling was necessary to arrest the process of RVD and keep the suitable difference of specific gravities between the cells and the silicone oil. The cells were separated from the media and precipitated below the oil by centrifugation at 1257 × g for 1.5 min at 4°C. They were collected and resuspended in 3 ml cold double-distilled water. A 2 ml part and a 1 ml part of each suspension were used for assaying the cations and protein, respectively. A preliminary test showed that deionized water was not contaminated with Na^+ of the silicone oil, after 1–2 ml of the water was passed through the silicone layer by centrifugation.

The cations were assayed by flamephotometry as reported in another paper [22]. Protein was determined by the method of Lowry et al. [23] using bovine serum albumin as the standard.

Reagents and other substances. Especially pure grade of inorganic salts and tetraethylammonium chloride (TEA) were purchased from Wako Pure; calf serum was from Nacalai Tesque; ouabain, quinine, furosemide, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM), bovine serum albumin (fraction V), *N*-2-hydroxyethylpiperadine-*N'*-2-ethanesulfonic acid (Hepes) were from Sigma; trypsin (1 : 250) was from Difco Laboratories; concentrated mixtures of amino acids (50 ×) and vitamins (100 ×) purchased from Gibco Laboratories were used to prepare the mMEM.

Results

Changes in cell volume, and the cellular contents of water and cations at different osmolalities

Upon incubation of attached HeLa cells in the hyposmotic medium of the osmolality of 235 mosmol/kg H_2O, the cellular water content increased by more than 30% in 2 min and then remained at the elevated

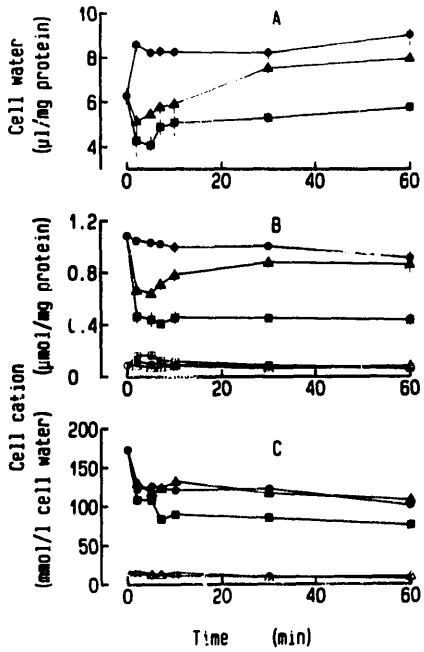

Fig. 1. Time-dependent changes in the contents of water and cations of HeLa cells attached to culture dishes after incubation in hyposmotic media of different osmolalities. (A) The water contents. ●, 235; ▲, 166; ■, 95 mosmol/kg H_2O. (B) The cation contents. (C) The cellular cation concentrations calculated from data shown in A and B. Symbols in B and C correspond to those in A, but closed and open symbols indicate K^+ and Na^+, respectively. Points and bars are means and S.D. values.

level for as long as 60 min (Fig. 1A). The cellular K^+ content seemed very slightly to decrease in 10 min, but the Na^+ content did not markedly alter (Fig. 1B). When the osmolality was decreased to 166 mosmol/kg H_2O, cellular water was lost. This was followed by slow gain of water, causing cell swelling in 30 min. In parallel with the initial loss of water, cellular K^+ decreased in 2 min, followed by its gain in 30 min. The Na^+ content did not change. We also studied the time-dependent change in cell volume semiquantitatively by microphotography (see Materials and Methods). Result shows that the cells started to swell 15 s after the medium replacement and attained a maximum volume at 20 s. The cell volume decreased to the normal level in 90 s and thereafter continued to shrink. When the osmolality was further decreased to 95 mosmol/kg H_2O, the cells lost water more markedly (Fig. 1B). The water content dropped by more than 30% and reached a minimum in 4 min and then increased until 10 min and thereafter the content approached the normal level in 60 min. Accompanied with the water loss, the cellular K^+ content significantly decreased in 2 min and then did not significantly change in 60 min. The Na^+ content seemed to increase slightly in 10 min. The cellular cation concentrations shown in Fig. 1C were calculated from the results in Figs. 1A and 1B. The cellular K^+ concentration decreased rapidly after the hyposmotic exposures and became steady after 2–8 min. The steady levels were about 120 mM and almost the same at 235 and 166 mosmol/kg H_2O, while the level was decreased to about 80 mM at 95 mosmol/kg H_2O. The attached cells seemed simply to swell in the medium of 235 mosmol/kg H_2O and behaved as ideal osmometers, judged from application of the cellular water content at the steady level to the Boyle-Van't Hoff equation. In contrast, the cells rapidly shrank and gained water later on in the more hyposmotic media of 166 and 95 mosmol/kg H_2O.

Upon incubation of suspended HeLa cells at 235 mosmol/kg H_2O, mean cell volume increased by about 23% to a peak value in 20 s and then decreased with time in 60 min to a level of slightly higher than the normal value (Fig. 2A). In the more hyposmotic medium of 166 mosmol/kg H_2O, cell volume instantly increased by about 50%, being about twice the increase at 235 mosmol/kg H_2O. Regulatory volume decrease (RVD) was more marked and cell volume was reduced to a similar level to that attained at 235 mosmol/kg H_2O in 60 min. Time required for the volume to attain the peak value somewhat delayed and the rate of RVD was slowed at 95 mosmol/kg H_2O. Applying the peak values of cell volume to the Boyle-Van't Hoff equation, we found that the suspended cells also behave like ideal osmometers at 235 mosmol/kg H_2O. However, the peak values at 166 and 95 mosmol/kg H_2O, were

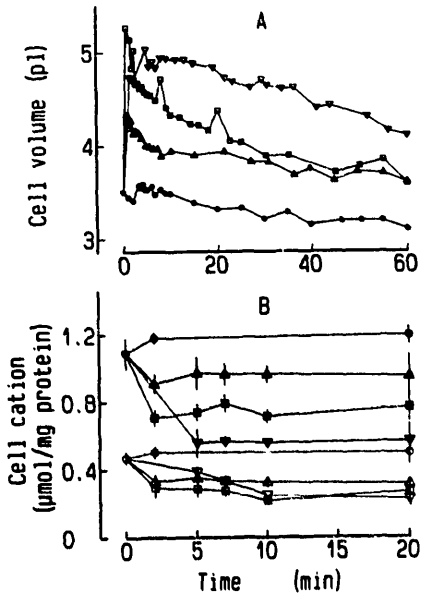

Fig. 2. Time-dependent changes in cell volume and the contents of cations of suspended HeLa cells after incubation in hyposmotic media of different osmolalities. (A) Cell volume. ○, 301; △, 235; □, 166; ▽, 95 mosmol/kg H_2O. (B) The cation contents. Symbols correspond to those in A, but closed symbols indicate the K^+ and open symbols the Na^+ contents. Points and bars are means and S.D. values.

far lower than those expected when assumed to be ideal osmometers. After replacement of the conditioned medium with the fresh isotonic medium, cell volume only slightly decreased in 60 min. Due to technical difficulties, we assayed cellular cations separately from cellular water. The cellular K^+ content was not significantly influenced by medium replacement with the isosmotic medium (Fig. 2B). The K^+ content decreased by about 17% in 2 min after exposure to the osmolality of 235 mosmol/kg H_2O, and then the content was unchanged. When incubated at 166 and 95 mosmol/kg H_2O, the K^+ contents dropped by about 35% and 50%, respectively, followed by staying at the reduced levels. The initial value of cellular Na^+ content was significantly higher than that of attached cells. This was an after-effect of trypsinization. The Na^+ content was unchanged upon incubation in the isosmotic medium, but was decreased with time after replacement with the hyposmotic media.

Because of the different patterns of changes in the cellular water contents at 166 mosmol/kg H_2O between attached and suspended cells (compare Figs. 1 and 2), we used only this medium in the following experiments.

Effects of transport inhibitors

Effects of 0.1 mM ouabain, 1 mM quinine and 10 mM TEA on cellular water and/or cation contents of attached cells incubated at 166 mosmol/kg H_2O were tested (Fig. 3). Initial rapid decrease and gradual re-

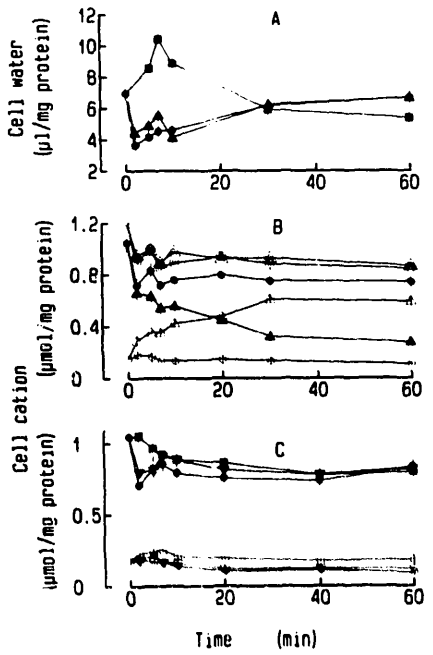

Fig. 3. Effects of inhibitors on time-dependent changes in the contents of water and cations of HeLa cells attached to culture dishes, after incubation in a hyposmotic medium of the osmolality of 166 mosmol/kg H_2O. (A) Effects of ouabain and quinine on the water contents. ●, control; ▲, 0.1 mM ouabain; ■, 1 mM quinine. (B) Effects of ouabain on the cation contents. Control: ●, K^+; ○, Na^+; ◯, K^+ plus Na^+. Ouabain (0.1 mM): ▲, K^+; △, Na^+; ◬, K^+ plus Na^+. (C) Effects of quinine and TEA on the cation contents. Control: ●, K^+; ○, Na^+. Quinine (1 mM): ■, K^+; □, Na^+. TEA (10 mM): ▼, K^+; ▽, Na^+. Points and bars are means and S.D. values.

covery of the water content of control cells shown in Fig. 1A were reproduced, but the final steady level was similar to the normal value in this case (Fig. 3A). Addition of ouabain did not significantly influence the water content (Fig. 3A), but the cellular K^+ content decreased and the Na^+ content increased with time (Fig. 3B). However, the sums of the Na^+ and K^+ contents that are known to control the cellular water contents were not significantly different from those of control cells. The rapid loss of cellular water was prevented by addition of quinine and the water was rather increased (Fig. 3A). The water content attained a maximum of about 50% higher than the normal value in 7 min, followed by decrease nearly to the normal value in 30 min. The rate of drop in the cellular K^+ content as observed for control cells was slowed down by the addition of quinine (Fig. 3C). Then, the K^+ content gradually decreased in 40 min nearly to the control level of about 25% below the normal value. The cellular Na^+ content was not markedly changed. Addition of TEA did not significantly influence the change in the cellular K^+ content and cellular Na^+ was not affected at all. Also, the changes in these cations were not influenced by addition of 0.2 mM furosemide (data not shown).

Changes in the water content and mean cell volume of the suspended cells were compared in Figs. 4A and B. The cells used in Fig. 4A were obtained by trypsinization of replicate cultures of those used in Fig. 3A. Hence, the experiments shown in Figs. 4A and B were made independently. Though experimental error was larger for determination of cell water than for assay of cell volume, the results clearly demonstrates a common pattern of changes in the two parameters, i.e.,: immediate increases after medium change to the hyposmotic medium followed by slow decreases. Different from the ineffectiveness of ouabain on the attached cells, addition of 0.1 mM ouabain significantly inhibited RVD. The ouabain-sensitive fraction of RVD was similar to the fraction sensitive to 1 mM quinine. Though these fractions were somewhat different in another experiment (data not shown), the sums of the two fractions covered nearly total RVD in the two experiments.

The sums of the K^+ and Na^+ contents showed the similar patterns of time-dependent changes between the suspended cells and the attached cells in the absence of inhibitor (compare Fig. 5 with Fig. 3B). Addition of quinine suppressed the decrease in cellular K^+ similarly to attached cells. Treatment with 0.1 mM ouabain also slowed the rate of decrease in the sums of both cations in the suspended cells, consistent with the delay of RVD (data not shown). The Na^+ content at

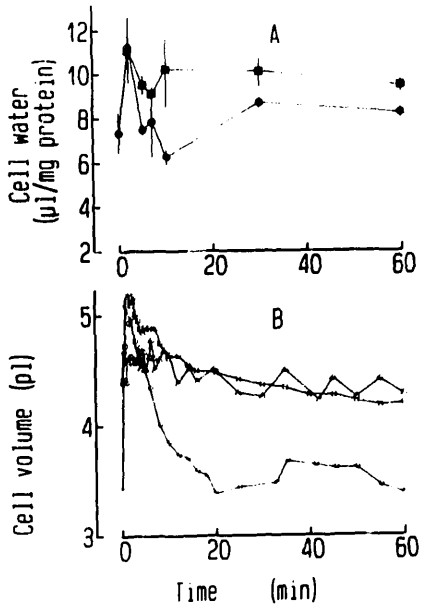

Fig. 4. Effects of inhibitors on time-dependent changes in the cellular contents of water and cell volume of suspended HeLa cells, after incubation in a hyposmotic medium of the osmolality of 166 mosmol/kg H_2O. (A) Effects of quinine on the water content. ●, control; ■, 1 mM quinine. The cells used in A was obtained by trypsin treatment of replicas of the cultures used in Fig. 3A. (B) Effects of ouabain and quinine on cell volume. ○, control; △, 0.1 mM ouabain; □, 1 mM quinine. Points and bars are means and S.D. values.

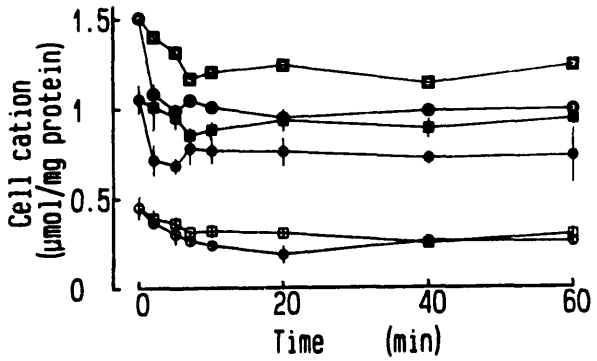

Fig. 5. Effects of quinine on time-dependent changes in the cellular cation contents of suspended HeLa cells after replacement with a hyposmotic medium (166 mosmol/kg H_2O). Control: ●, K^+; ○, Na^+; ◎, K^+ plus Na^+. Quinine (1 mM): ■, K^+; □, Na^+; ▣, K^+ plus Na^+. Points and bars are means and S.D. values.

time 0, i.e., before the medium replacement, was again relatively high in suspended cells as in the present case.

Effects of BAPTA

We tested the effects of Ca^{2+}-deprivation from the medium and/or cytoplasm and Ba^{2+}. For these purposes, we added 10 mM $BaCl_2$ to the hyposmotic medium (166 mosmol/kg H_2O) or a permeable Ca^{2+} chelating agent BAPTA-AM (10 μM) to the Ca^{2+}-free medium of the same osmolality.

Simple removal of Ca^{2+} from the medium in the presence of 1 mM EGTA partially inhibited the rapid decrease in the K^+ content of the attached cells (Fig. 6). Addition of BAPTA or $BaCl_2$ suppressed the initial rapid decrease completely.

RVD of the suspended cells in Ca^{2+}-free medium in the presence of 1 mM EGTA was significantly suppressed (Fig. 7A). Addition of 10 μM BAPTA to the medium did not enhance the inhibition in the Ca^{2+}-free medium any further. The similar inhibition was observed upon addition of 10 mM $BaCl_2$ to the medium containing Ca^{2+} (Fig. 7B).

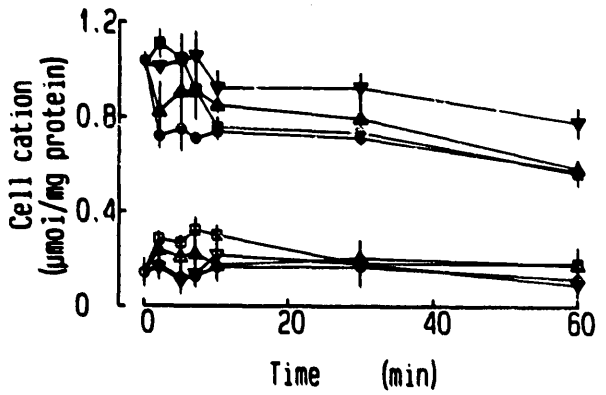

Fig. 6. Effects of addition of $BaCl_2$, omission of medium Ca^{2+} and addition of BAPTA to the Ca^{2+}-free medium on time-dependent changes in the cation contents of HeLa cells attached to culture dishes, after incubation in a hyposmotic medium. The medium contained 1 mM EGTA in the absence of Ca^{2+} and its osmolality was 166 mosmol/kg H_2O. Control: ●, K^+; ○, Na^+. $BaCl_2$ (10 mM): ▼, K^+; ▽, Na^+. Ca^{2+}-deficiency: ▲, K^+; △, Na^+. BAPTA-AM (10 μM): ■, K^+; □, Na^+. Points and bars are means and S.D. values.

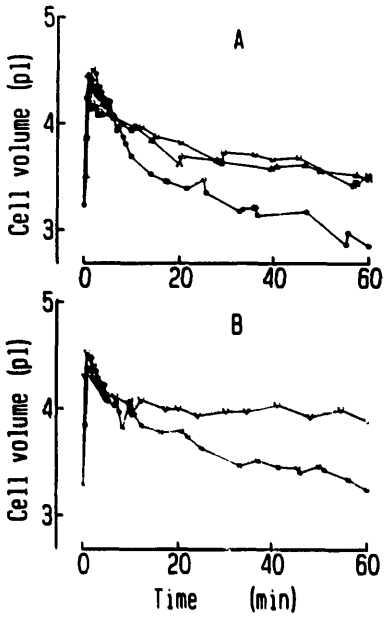

Fig. 7. Effects of omission of medium Ca^{2+} and addition of BAPTA to the Ca^{2+}-free medium on time-dependent changes in cell volume of suspended HeLa cells, after incubation in a hyposmotic medium (166 mosmol/kg H_2O). (A) Effects of omission of medium Ca^{2+} and addition of BAPTA. ○, control; △, 1 mM EGTA in the absence of Ca^{2+}; □, BAPTA-AM (10 μM). (B) Effects of addition of $BaCl_2$. ○, control; ▽, 10 mM $BaCl_2$.

Discussion

Different patterns of time-dependent volume changes between attached and suspended HeLa cells were found upon exposure to hyposmotic conditions.

Attached HeLa cells

Attached HeLa cells incubated in the hyposmotic medium of 235 mosmol/kg H_2O instantly gained water and behaved like ideal osmometers without any further change in the water content. The occurrence of cell swelling has been reported for suspended HeLa cells incubated in a medium of the same osmolarity [18]. However, the reported cells do not behave as the osmometers in the hyposmotic media. Rapid increase in cell height corresponding to the gain of water occurs in monolayer of MDCK cells by incubation in a more hyposmotic medium of 125 mosmol/kg H_2O, and any decrease in the height is not observed for following 20 min [24]. However, when the osmolality was decreased to 166 mosmol/kg H_2O or less, instant cell swelling took place; which was followed by simultaneous decreases in cellular water and K^+. This resembles K^+

release reported for attached HeLa cells in a hypotonic medium [18]. The decreases were followed by their gradual increases seemingly like RVI.

Addition of quinine did not only prevent the shrinkage but even caused swelling of the cells, which was accompanied by marked inhibition of K^+ loss from the cells. The same inhibition of K^+ loss was shown in the Ca^{2+}-free medium especially on addition of BAPTA to the medium or simple addition $BaCl_2$. But, any inhibition of cell shrinkage was not detected when treated with ouabain, TEA or furosemide. These results imply that attached HeLa cells are expected to gain water due to the difference of osmotic pressure in any hyposmotic media. But the gain is overcome by water loss associated with K^+ efflux through activated Ca^{2+}-dependent K^+ channel at the osmolality of 166 mosmol/kg H_2O or less, resulting in net loss of cellular water. The mechanism of the loss of water is similar to that of the secondary active transport of water reported for choroid plexus epithelium of *Necturus* [25]. The K^+ efflux would depend on the transmembrane gradient of electrochemical potential of K^+. A considerably greater swelling of the perfused rat bil proximal nephron has been reported in the presence than the absence of quinine after hyposmotic stress [12].

The activation of the K^+ channel of attached HeLa cells by the hyposmotic stress would be transient and does not persist, so that entry of water begins after the initial shrinkage driven by the gradient of osmotic pressure like an oscillatory phenomenon. The inward water movement secondarily induces K^+ influx. Therefore, the volume increase in the hyposmotic media takes place by entirely different mechanisms from those of RVI reported for other cells in hyperosmotic media [15,26]. Because, RVI is closely related with Na^+ influx.

Suspended HeLa cells

The suspended cells showed immediate increase in cell volume upon incubation in the hyposmotic media. The increased cell volume gradually decreased with time. The peak value of cell volume at the osmolality of 235 mosmol/kg H_2O suggested that the cells behave as ideal osmometers like the attached cells. The increase in cell volume was greater in the media of 166 and 95 mosmol/kg H_2O. However, the cells did not behave as the osmometers at the lower osmolarities. Their volumes were smaller than those expected when they behave as ideal osmometers. The incomplete swelling at 166 mosmol/kg H_2O could not be attributed to K^+ efflux mediated by the K^+ channel, because addition of quinine had no influence to the peak value of cell volume. Loss of K^+ and RVD occurred after the cell swelling, which were significantly inhibited by Ca^{2+}-deprivation and addition of quinine. However, addition of BAPTA-AM did not enhance the inhibition by deprivation of medium Ca^{2+}, implying that deficiency of medium Ca^{2+} sufficiently decreases intracellular Ca^{2+}. Addition of ouabain inhibited RVD. Therefore, Na^+-pump also participates in RVD of the suspended cells. The similar ouabain inhibition of RVD has been reported for the rabbit thin descending limb of Henle's loop [27].

These results suggest that hyposmotic exposure of suspended HeLa cells induces rapid gain of water due to the transmembrane gradient of osmotic pressure, resulting in cell swelling. Then, K^+ efflux may be stimulated as found in other cells [5,6,9,28,29]. However, K^+ efflux would be insufficient to overcome the cell swelling in the suspended cells. The activation of K^+ efflux by hyposmotic stress has been reported for chicken enterocytes [29] and isolated rat liver [30]. Human lymphocytes also show activation of net K^+ release [1]. Moreover, the relatively high initial Na^+ concentration of suspended HeLa cells as an aftereffect of trypsinization must stimulate Na^+-pump and increase Na^+ release [22]. The sums of the parts of RVD sensitive to quinine and ouabain seemed almost to equal total RVD.

Comparison of the attached and suspended HeLa cells

We consider that the distinctive patterns of volume changes between attached and suspended HeLa cells are mainly based on the difference of activity or time of activation of the K^+ channel upon hyposmotic exposure. The activation of the K^+ channel would be quicker and stronger in attached than in suspended HeLa cells. This can be noticed from larger quinine-sensitive loss of water from the attached cells than from the suspended cells as shown in Figs. 3A and 4A. The underlying mechanism(s) causing the difference is not known. Hyposmotic stress given to extended surface of attached cells might relate to the rapid and strong activation of the channel, based on the larger number of active K^+ channels than suspended cells. Stretch of the patch membrane of medullary ascending limb cells by negative pressure has been reported to increase the open probability of Ca^{2+}-activated K^+ channel [31]. Therefore, the number of active channels may be larger in the attached cells, which would induce instant K^+ release and cell shrinkage upon hyposmotic exposure. In contrast, K^+ release may start after swelling due to stretch of the cell membrane in the suspended cells, resulted in RVD. RVD have been reported in various types of suspended cells. These cells include mouse ascites tumor cells [4] and human lymphocytes [1,2], which are naturally suspended in media, implying that the pattern of RVD is a common phenomenon in suspended cells and not necessarily be attributed to trypsin treatment. However, a possibility of partial inactivation of the K^+ channel by the treat-

ment cannot fully be ignored. Stimulation of Na^+-pump possibly related to the increased cellular Na^+ concentration may also contribute RVD of the suspended cells.

Attached HeLa cells and peak value of cell volume of suspended HeLa cells suggested that they behaved as ideal osmometers at the osmolality of 235 mosmol/kg H_2O, but they markedly lost K^+ in the more hyposmotic medium of 166 mosmol/kg H_2O or less. Therefore, the osmolality thresholds with respect to activation of the K^+ channels may not so differ between both cells, regardless of the differences of culture conditions and activity of the channels.

Finally, we came to the following conclusions. Simple swelling of attached HeLa cells occurred in a hyposmotic medium at the osmolality of 235 mosmol/kg H_2O. When the medium osmolality was reduced to 166 mosmol/kg H_2O or less, the cells instantly swelled and then rapidly shrank. Following the shrinkage, cell volume started to increase. Suspended HeLa cells showed transient swelling, followed by RVD. The distinctive patterns of cell volume change may depend on the difference of either activity or time of activation of Ca^{2+}-dependent K^+ channel between the attached and suspended cells. Stimulation of Na^+-pump may also contribute RVD of the suspended cells.

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