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Regulatory changes in the K^+, Cl^- and water contents of HeLa cells incubated in an isosmotic high K^+-medium

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HeLa cells had their normal medium replaced by an isosmotic medium containing 80 mM K^+, 70 mM Na^+ and 100 μM ouabain. The cellular contents of K^+ first increased and then decreased to the original values, that is, the cells showed a regulatory decrease (RVD) in size. The initial increase was not inhibited by various agents except by substitution of medium Cl^- with gluconate. In contrast, the regulatory decrease was inhibited strongly by addition of either 1 mM quinine, 10 μM BAPTA-AM without medium Ca^{2+}, or 0.5 mM DIDS, and partly by either 1 mM EGTA without medium Ca^{2+}, 10 μM trifluoperazine, or substitution of medium Cl^- with NO_3^-. Addition of DIDS to the NO_3^--substituted medium further suppressed the K^+ loss but the effect was incomplete. Intracellular Ca^{2+} showed a transient increase after the medium replacement. These results suggest that the initial increase in cell K^+ is a phenomenon related to osmotic water movement toward Donnan equilibrium, whereas the regulatory K^+ decrease is caused by K^+ efflux through Ca^{2+}-dependent K^+ channels. The K^+ decrease induced a decrease in cellular water, i.e., RVD. The K^+ efflux may be more selectively associated with Cl^- efflux through DIDS-sensitive channels than the efflux of other anions.

Introduction

It has been reported that human red cells incubated in hyper- and hypo-osmotic media change their volume simply dependent on osmolarity of the media and behave like ideal osmometers [1]. HeLa cells placed in a hyperosmotic medium also behave like osmometers but their swelling in hyposmotic media is unexpectedly weak [2]. On the other hand, many other types of cells are known to show rather complicated processes of volume change and in certain cases return to their original cell volumes, provided that the cells are incubated in anisosmotic culture media. For example, red cells of duck [3], *Amphiuma* [4] and rat [1] placed in hyperosmotic media rapidly shrink, and thereafter gradually restore their original cell volumes; i.e., they show regulatory volume increase (RVI). Other types of cells, however, do not show such volume increase in hyperosmotic media. Human lymphocytes [5] and Ehrlich ascites tumor cells [6] simply shrink in hyperosmotic media and their normal volume is recovered after return to isosmotic media. The initial cell shrinkage is due to outward water movement induced osmotically, but RVI is accompanied by activation of pathways for transmembrane ion transport. The reswelling in RVI seemed to relate with net influx of ions especially of Na^+ and Cl^-. In contrast, various types of cells placed in hyposmotic media rapidly swell and thereafter their volumes gradually return to normal values. i.e., they show regulatory volume decrease (RVD). These cells comprise cells of renal tubules isolated from rabbit kidney [7,8], *Amphiuma* red cells [9], Ehrlich ascites tumor cells [10,11], chicken red cells [12] and human lymphocytes [13]. The initial rapid swelling is caused by water entry into the cells driven by the transmembrane gradient of osmotic pressure, whereas RVD seemed chiefly to be associated with effluxes of K^+ and Cl^-, as judged by the effects of transport inhibitors.

While thymocytes are reported to maintain original cell volumes in an isosmotic high K^+-medium contain-

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ing 70 mM K^+ and 70 mM Na^+ [14], we show in the present study that the contents of water, K^+ and Cl^- of HeLa cells initially increase and then gradually decrease to nearly normal levels, when exposed to a similar medium, e.g., 70 mM Na^+ and 80 mM K^+. This change is very similar to RVD observed for other types of cells incubated in hyposmotic media, except that time required for the change is much longer than that reported for the RVD. It may be caused by a mechanism common to what induces smaller swelling of HeLa cells in hyposmotic media than that expected if the cells are assumed to be ideal osmometers [2].

In the present study, we intend to clarify the mechanisms of changes in the ion and water contents of HeLa cells incubated in the isosmotic high K^+-medium. Especially, pathways of transmembrane K^+ transport closely related to the volume changes are investigated in connection with changes in the cellular Ca^{2+} concentration. Coupling of Cl^- and K^+ transports during the changes is also investigated.

Materials and Methods

Cell cultures

HeLa S3 cells purchased from Flow Laboratories were serially cultivated in glass culture flasks containing 10 ml of modified Eagle's minimum essential medium supplemented with 10% (v/v) calf serum [15]. Confluent cultures of the cells were washed once with the same medium and dispersed with 0.5% trypsin (1:250, Difco). The cells were then suspended and inoculated at a density of $3 \cdot 10^4$ cells/ml in Roux flasks containing 100 ml of the medium. After 96 h of incubation, growing cultures of the cells were resuspended by tryptic digestion and reinoculated in plastic culture dishes (6 cm diameter, Corning) containing 5 ml of the culture medium at a density of $7 \cdot 10^4$ cells/ml. These dishes were placed for further 40 h at 37° C in a CO_2 incubator in an atmosphere of 5% CO_2 in humid air.

Experiments

The cultures were washed and the culture medium was replaced with similar media but containing different mixtures of Na^+ and K^+ shown in the Results. The counter anion for these cations, i.e., normally Cl^-, was changed with the same concentration (150 mM) of another permeable or impermeable anion as NO_3^- or gluconate when necessary. Serum was omitted from these media but 100 μM ouabain and 20 mM Hepes buffer (pH 7.2) were added. Immediately after the medium replacement, the cultures were placed in an incubator exactly at 37° C in humid air for various periods indicated in the Results. The cultures were taken out of the incubator at the end of the incubation periods and washed six times with chilled 150 mM $LiNO_3$ in 15 s by the method reported elsewhere [16]. Thereafter, 3.5 ml of chilled deionized and distilled water was put into each culture dish and the cells were scraped off and suspended in the water by a rotating silicon-rubber policeman. A 1 ml part of the cell suspension was mixed with 1 ml of 1 M NaOH for protein assay. Another part of 2 ml was placed at room temperature at least overnight to lyze the cells completely.

Assays

The 2 ml part of the cell suspension was added with the same amount of 15 mM $LiNO_3$ as the internal standard and used for assay of alkali cations by flamephotometry as reported previously [16]. The cellular contents of the cations were normally expressed as μmol/mg of cell protein.

Protein was determined with Folin-phenol reagent by the method of Lowry et al. [17]. Bovine serum albumin (fraction V) was used as the standard.

Osmotic pressures of the culture and incubation media used for experiments were measured by an automatic osmometer (Osmostat OM-6020, Kyoto Daiichi Kagaku).

The concentration of intracellular free Ca^{2+} was determined by microfluorometry of a fluorescent dye 1-(2-(5'-carboxyoxazol-2'-yl)-6-aminobenzofuran-5-oxy)-2-(2'-amino-5'-methylphenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid, pentaacetoxy methyl ester (fura-2/AM) at excitation wavelengths of 340 nm and 380 nm and a emission wavelength of 510 nm, using a specially designed flow chamber. Medium replacements in this chamber were completed in about 1 min. As the culture medium before medium replacement contained calf serum, mean background emission from the cell preparations without fura-2 was subtracted from mean emission of the fura-2-loaded preparations to minimize artifacts mainly due to emission from the serum.

The cellular water content was determined with [^{14}C]urea (specific activity 56 Ci/mol, Amersham). To equilibrate the intra- and extracellular concentrations of the radio active urea, the cells attached to the plastic culture dishes were preincubated at 37° C for 30 min with 2 ml of the culture medium containing 1 μCi/ml of the radioactive agent by the method of Vasquez et al. [18]. Then, the culture medium was replaced with the high K^+-medium containing 70 mM Na^+, 80 mM K^+ and the same activity of [^{14}C]urea as in the preincubation medium. Samples of the labeled cells were taken at the time points indicated in the Results and quickly washed six times with more than 2 ml of the cold isosmotic $LiNO_3$. The protein content of the samples were determined using replicate cultures. Radioactivity was measured with a liquid scintillation spectrometer (LSC-62, Aloka). The cellular water content was calculated assuming that the intra- and extracellular concentrations of the radioactive urea were

equilibrated and that change in the cellular content of the urea is consistent with change in the water content after the medium replacement. The water content was finally expressed as μl/mg of cellular protein.

The cellular Cl^- contents shown in Table I were determined by amperometry using NaCl as standard as described elsewhere [19], but the contents shown in Fig. 7 were assayed after equilibration for 30 min with 0.2 μCi/ml of $^{36}Cl^-$ (3 mCi/g of Cl, Amersham) contained in the culture medium. Changes in the Cl^- content with time were pursued after medium replacement with the isosmotic high K^+-medium containing the same concentration of the isotope. Also, loss of Cl^- from the cells was chased after washing the culture dishes with the same replacing medium that did not contain the isotope. The procedures for measuring radioacativity were the same with those described for [^{14}C]urea.

Reagents and other substances

Guaranteed grade of inorganic salts were purchased from Wako; ouabain, quinine, furosemide, ionomycin, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), trifluoperazine, tetraethylammonium (TEA), and *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (Hepes), were from Sigma; sodium and potassium gluconate and calf serum were from Nakarai; superpure rubidium chloride was from Merck; and 2',7'-bis(carboxyethyl)carboxyfluorescein tetraacetoxy methyl ester (BAPTA-AM) and glycoletherdiamine-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) were from Dojindo. Amiloride was given from Merck Sharp and Dohme Co., Inc. For convenience, concentrated mixtures of amino acids (50 ×) and vitamins (100 ×) purchased from Gibco were used for preparing the modified minimum essential medium.

Results

Osmotic pressures of media

The osmotic concentrations of media used in the present study were determined. The media contained various concentrations of Na^+ and K^+ but their sums were kept constantly at 150 mM. Results were as follows: 150 mM K^+ without Na^+, 305 ± 7 mosM (n = 3); 80 mM Na^+ and 70 mM K^+, 298 ± 5 mosM (n = 4); 145 mM Na^+ and 5 mM K^+, 301 ± 6 mosM (n = 3); 150 mM Na^+ without K^+, 305 ± 11 mosM (n = 2). For comparison, the osmotic concentration of the conditioned culture medium was assayed, being 305 ± 1 mosM (n = 3). These results showed isosmocity of all of the tested media.

Changes in the cation contents after medium replacement

The effects on the cellular ion contents of medium replacement from the normal culture medium to medium without serum and of identical ion composition in the presence and absence of ouabain are shown in Table I. There were no significant time-dependent changes in the cellular of Na^+, K^+ and Cl^- in the absence of ouabain. When ouabain was added, the Na^+ content increased but K^+ decreased with time, keeping the sums of the two cation contents almost unchanged. The Cl^- contents were not significantly altered regardless of the addition of ouabain.

TABLE I

Changes in the cellular ion contents after replacement of the normal culture medium to a medium with the same ion composition but containing 100 μM ouabain without serum

Means and S.D. values are for four samples.

Time (min)	Ouabain	μmol/mg of cell protein			
		Na^+	K^+	Cl^-	$Na^+ + K^+$
0	–	0.165 ± 0.031	0.970 ± 0.033	0.576 ± 0.050	1.135
10	–	0.143 ± 0.040	0.992 ± 0.022	0.595 ± 0.014	1.135
	+	0.167 ± 0.023	0.939 ± 0.029	0.599 ± 0.043	1.106
20	–	0.144 ± 0.028	0.989 ± 0.035	0.578 ± 0.089	1.133
	+	0.234 ± 0.026	0.931 ± 0.019	0.564 ± 0.054	1.165
30	–	0.150 ± 0.006	1.006 ± 0.044	0.580 ± 0.079	1.156
	+	0.275 ± 0.012	0.892 ± 0.033	0.573 ± 0.074	1.167
60	–	0.152 ± 0.036	0.988 ± 0.027	0.597 ± 0.036	1.140
	+	0.330 ± 0.025	0.817 ± 0.029	0.558 ± 0.076	1.147

Changes in the cellular cation contents with time after medium replacement from the culture medium to isosmotic high K^+-media are shown in Fig. 1. The high K^+-media contained different concentrations of Na^+ and K^+ but keeping their sums at 150 mM in the presence of 100 μM ouabain. The cellular K^+ content in the medium containing 150 mM K^+ without Na^+ increased in 30 min to a level of about 2.5-times the original (normal) one and thereafter the content remained almost unchanged until 60 min (Fig. 1A). When the cells were placed in the medium with 25 mM Na^+ and 125 mM K^+, the K^+ content initially increased and attained a constant level of about 1.5 times the original one from 20 min on, suggesting that K^+ influx was offset by its efflux. Upon replacement to the medium containing 50 mM Na^+ and 100 mM K^+, the K^+ content seemed slightly to decrease from 10 min following the initial rise. The delayed decrease in the K^+ content became more significant when medium K^+ concentration was reduced to 70 mM or less (Fig. 1B). Cellular Na^+ was almost lost in 10 min provided that the medium K^+ concentration was higher than 100 mM (Fig. 1A), while the loss was smaller when the concentration was below 80 mM.

Effects of quinine

The transient increase and delayed decrease in the cellular K^+ content were found later on to be more highly reproducible when an isosmotic medium con-

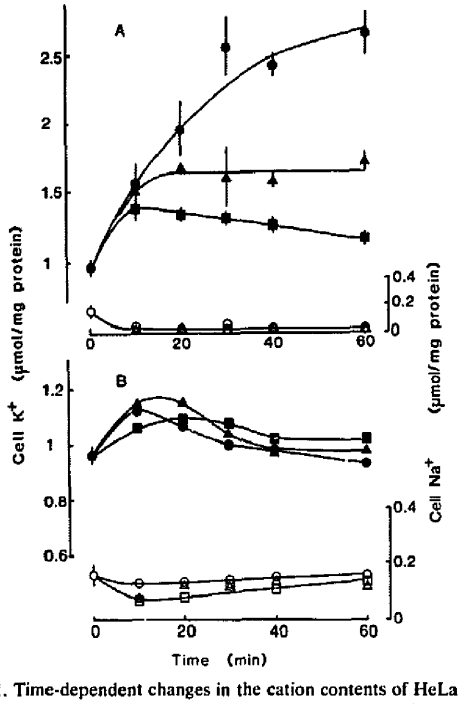

Fig. 1. Time-dependent changes in the cation contents of HeLa cells after medium replacement with isosmotic media containing various concentrations of K^+ and Na^+ and 100 μM ouabain. (A) The K^+ contents. ●, 150 mM K^+ only; ▲, 125 mM K^+ and 25 mM Na^+; ■, 100 mM K^+ and 50 mM Na^+. (B) The K^+ contents. ■, 70 mM K^+ and 80 mM Na^+; ▲, 60 mM K^+ and 90 mM Na^+; ●, 40 mM K^+ and 110 mM Na^+. The Na^+ contents are represented by the same but open symbols. Points and bars are means and S.D. values for four samples.

taining 70 mM Na^+, 80 mM K^+ and 100 μM ouabain (80 mM K^+-medium) was used. After medium replacement with the medium, the cellular K^+ content initially increased and reached a peak of 14% higher than the initial value at 10 min. Then, the content started to decrease, i.e., regulatory K^+ decrease (Fig. 2). The rate of decrease became slower after 30 min. The cellular Na^+ content decreased only slightly, so that the sums of the Na^+ and the K^+ contents returned nearly to the original level from 30 min on. When 100 μM ouabain was added to the medium, the cellular K^+ content became slightly smaller than before, but the pattern of its time-dependent change was similar. As the Na^+ content increased only slightly, the sums of the Na^+ and K^+ contents became the same with that observed in the absence of ouabain after 30 min. A further addition of an inhibitor of Ca^{2+}-dependent K^+ channel quinine (1 mM) did not influence the initial rise in the K^+ content after the medium change, but suppressed its regulatory decrease. Cellular Na^+ slightly increased after the medium replacement, irrespective of the presence of quinine. Hence, the sums of the Na^+ and K^+ contents stayed at a level of 12% higher than the normal one in the presence of quinine, suggesting almost complete suppression of RVD. In contrast, the sums of the cation contents decreased nearly to the original level in less than 60 min, when quinine was omitted. Since Na^+-pump was proved not to affect the regulatory K^+ decrease, medium was replaced with the 80 mM K^+-medium, unless especially described.

K^+ uptake and loss

To determine cellular K^+ uptake and loss after the medium replacement, K^+ in the 80 mM K^+-medium was totally substituted with Rb^+. By this substitution, the K^+ uptake could be represented by Rb^+ accumulation and K^+ loss by a decrease in the K^+ content with time. The loss began 10 min after the medium replacement and seemed to be slowed in the presence of 1 mM quinine (Fig. 3A). We plotted data of Fig. 3A in Fig. 3B according to a logarithmic expression for estimating and comparing rate constants of the K^+ loss. Fig. 3B reveals that the difference of the rate constants was insignificant between control and quinine-treated cells until 25 min, but that thereafter it became significant. As the original K^+ content was 1.068 μmol/mg of cell protein, K^+ losses are estimated to be 19.5 and 31.6 nmol/mg of cell protein/min until 25 min and from 30 min, respectively, in control cells. The loss was to be 14.5 nmol/mg per min in quinine-treated cells. Rb^+ uptake started immediately after the medium replacement (Fig. 3C). The uptake took place at initial rates of 21.3 and 18.5 nmol/mg of cell protein/min for 30 min in control and quinine-treated cells, respectively. Hence, there were no signifi-

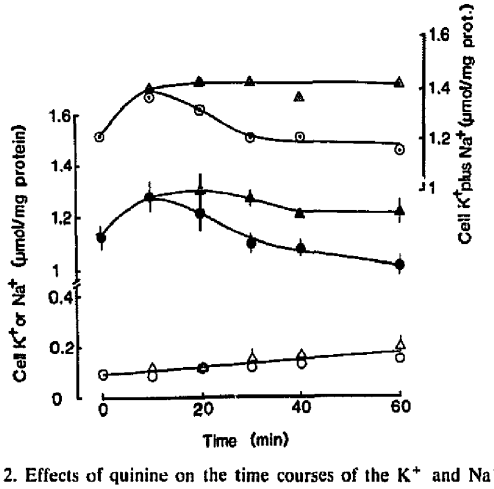

Fig. 2. Effects of quinine on the time courses of the K^+ and Na^+ contents of HeLa cells after medium replacement with a medium containing 80 mM K^+, 70 mM Na^+ and 100 μM ouabain. The K^+ contents. ●, control; ▲, 1 mM quinine. The Na^+ contents. ○, control; △, 1 mM quinine. The sums of the K^+ and Na^+ contents. ⊙, control; ▲, 1 mM quinine. Points and bars are means and SD's for four samples.

cant difference of the rates between the two groups of cells. These results indicate that the rates of K^+ loss in control cells within 25 min and in quinine-treated cells are similar to the rate of Rb^+ uptakes in both control and quinine-treated cells. But, K^+ loss in control cells after 30 min is significantly greater than Rb^+ uptake in the same cells. The K^+ losses and Rb^+ uptake are reflected on the changes in the sums of the cellular K^+ and Rb^+ contents shown in Fig. 3D. Namely, the sums increased by about 20% and attained a maximum at 10 min, which were maintained until 25 min. Then, the sum returned nearly to the original K^+ level in 60 min in control cells, whereas it remained unchanged in the presence of quinine. The pattern of change indicates regulatory decrease in the sum of the K^+ and Rb^+ contents and its inhibition by quinine, being the same with the change in the K^+ content shown in the absence of Rb^+.

Change in the cellular water contents

Time-dependent change in the cellular water content after the same medium change was also tested (Fig. 4). The water content initially increased and reached a maximum of 43% higher than the original value at 10 min, followed by a decrease to the levels of 9% above the latter value at 30 min and only 6% at 60 min. The maximum value at 10 min is significantly higher than the original value ($P < 0.01$) but values after 20 min are insignificantly higher ($P > 0.05$). The results demonstrated that RVD took place in HeLa cells incubated in the 80 mM K^+-medium. Though the

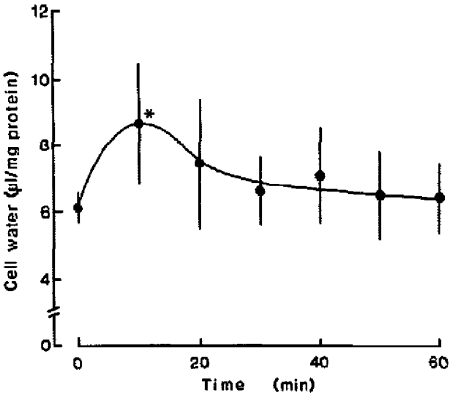

Fig. 4. Time-dependent change in the water content of HeLa cells after medium replacement with a medium containing 80 mM K^+, 70 mM Na^+ and 100 μM ouabain. The water contents were measured with [^{14}C]urea. Points and bars are means and S.D. values for 6–8 samples. *, significantly different from value at time zero ($P < 0.01$).

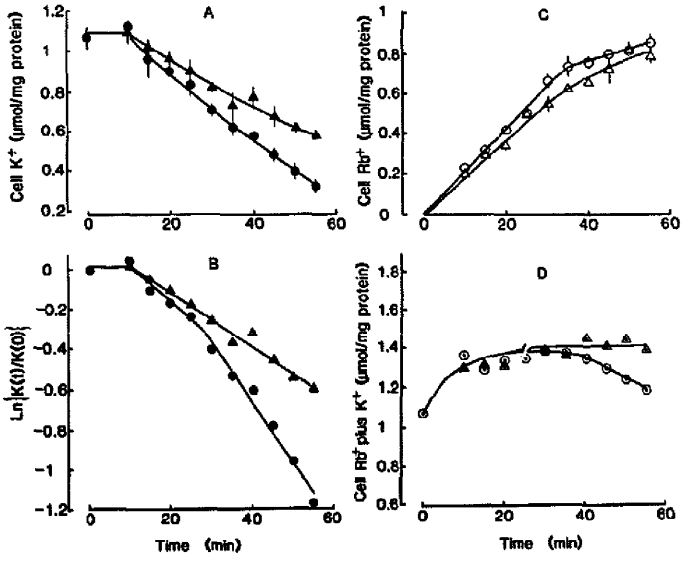

Fig. 3. Effects of quinine on time-dependent changes in K^+ loss and Rb^+ uptake of HeLa cells after medium replacement with a medium containing 80 mM Rb^+, 70 mM Na^+ and 100 μM ouabain. Circles indicate control and triangles addition of 1 mM quinine. (A) Loss of cell K^+. (B) Logarithmic plot of data in A. The slopes of the regression lines, i.e., the rate constants of K^+ loss, were $k = 0.0183$/min and $k = -0.0296$/min for control until 25 min and from 30 min on, respectively, which were significantly different ($P < 0.05$, t-test). For 1 mM quinine, the rate constant was $k = -0.0136$/min after 10 min. The constants for control from 30 min and that for quinine were significantly different ($P < 0.01$, t-test). The original cellular K^+ content was 1.068 μmol/mg of cell protein. (C) Rb^+ uptake. The slopes of the regression lines until 30 min, i.e., the initial rates, were 21.3 and 18.5 nmol/mg protein per min for control and 1 mM quinine. The initial rates were not significantly different ($P > 0.05$, t-test). (D) The sums of the K^+ and Rb^+ contents. Points and bars are means and S.D. values for four samples.

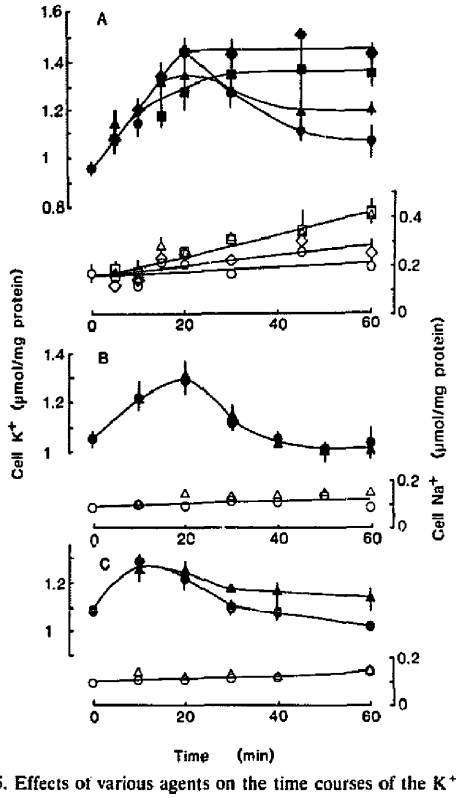

Fig. 5. Effects of various agents on the time courses of the K^+ and Na^+ contents of HeLa cells after medium replacement with a medium containing 80 mM K^+, 70 mM Na^+ and 100 μM ouabain. (A) Effects of BAPTA-AM and EGTA. The 80 mM K^+-medium contained these chelating agents without Ca^{2+}. Only the medium for control and quinine contained 1.8 mM Ca^{2+}. The K^+ contents. ●, control; ■, 10 μM BAPTA-AM; ▲, 1 mM EGTA; ◆, 0.5 mM quinine. (B) Effect of ionomycin. The K^+ contents. ●, control; ▲, 1 μM ionomycin. (C) Effect of trifluoperazine. ●, control; ▲, 10 μM trifluoperazine. The Na^+ contents are represented by the same but open symbols in all cases. Points and bars are means and S.D. values for four samples.

experiments shown in Figs. 2, 4, 5, 7 and 8 were carried out separately, time courses of the changes in the K^+, Cl^- and water contents per mg of cell protein were similar. Hence, the intracellular concentrations of K^+ and Cl^- expressed per μl of cell water would not significantly change within the experimental errors after medium replacement.

Effects of agents influencing cellular Ca^{2+}

Addition of a permeable Ca^{2+}-chelating agent BAPTA-AM (10 μM) to the Ca^{2+}-free 80 mM K^+-medium slightly suppressed a latest part of the initial increase in the K^+ content, whereas the regulatory K^+ decrease was inhibited completely as understood when compared with the effect of quinine (Fig. 5A). When tracer amount of Ca^{2+} in the Ca^{2+} free 80 mM K^+-medium was removed by addition of 1 mM EGTA, the decrease in the K^+ content was inhibited in part, i.e., 12% higher than the control level at 60 min. In both cases, there was a tendency of weak stimulation of increase in the cellular Na^+ content, as usually observed on omission of medium Ca^{2+}. Activation of forward mode of Na^+–Ca^{2+} exchange might also be related in part [20]. The increase in cellular Na^+ implies weak cell swelling. Addition of 1 μM ionomycin to the 80 mM K^+-medium containing 1.8 mM Ca^{2+} did not significantly influence both the initial increase and the regulatory decrease in the K^+ content (Fig. 5B). Therefore, any increase in the cellular Ca^{2+} concentration exceeding that attained after the medium change is not necessary to cause the regulatory changes in the K^+ and water contents.

The effect of a calmodulin inhibitor trifluoperazine (10 μM) on the K^+ content after the medium change was compared with the effect of quinine (Fig. 5C). The addition of trifluoperazine did not influence the initial rise in the K^+ content but inhibited its regulatory decrease by 11% at 60 min compared to control, the inhibition being less strong than quinine. The cellular Na^+ content was not significantly affected by the inhibitor.

Change in the cellular Ca^{2+} concentration

Time courses of change in the cellular free Ca^{2+} concentration after medium replacement from conditioned culture medium to the normal and the 80 mM K^+-medium were examined (Fig. 6). The Ca^{2+} concentration began to decrease after the start of medium replacement to the normal medium and reached a minimum of about 170 nM in 4 min. The decrease in the Ca^{2+} concentration was probably due to washout of the remnant fluorescent dye bound to serum protein by flowing the serum-free media. Then, it began to increase insignificantly and attained a steady level of 220 nM in 15 min. This medium replacement did not cause any significant changes in the cellular cation contents (data not shown). Conversely, immediately after the start of replacement to the 80 mM K^+-medium, the Ca^{2+} concentration rapidly increased and attained a maximum of 520 nM in less than 2 min, and then decreased to about 250 nM in 4 min. Thereafter, it again slowly increased and reached a supranormal steady level in less than 15 min. To minimize artifacts and determine net changes in cellular Ca^{2+} triggered by replacement to the high K^+-medium, the difference of the two cellular Ca^{2+} concentrations stated above was calculated and its time course is shown in the inset of Fig. 6. The difference revealed that the Ca^{2+} concentration showed a fast increase immediately after the start of the medium replacement. The increase was followed by a sudden decrease and there was a second phase of slow increase in 10 min.

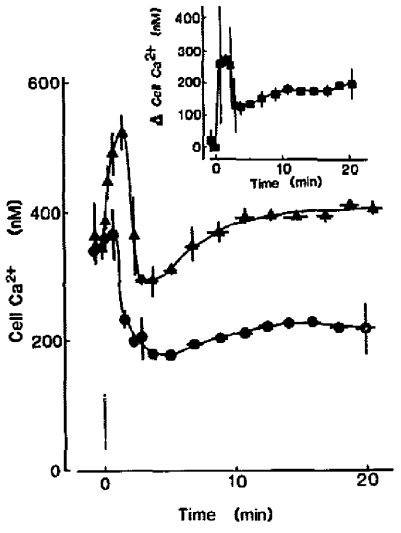

Fig. 6. Time-dependent changes in the intracellular Ca^{2+} concentration after medium replacement with the normal medium or the 80 mM K^+-medium. The medium replacement started at time zero as indicated by an arrow and was completed after about 1 min. ●, control; ▲, the 80 mM K^+-medium. Points and bars are means and S.D. values for eight samples. Inset: plots of the differences between values for the normal medium and those for the 80 mM K^+-medium. See also the Materials and Methods for details.

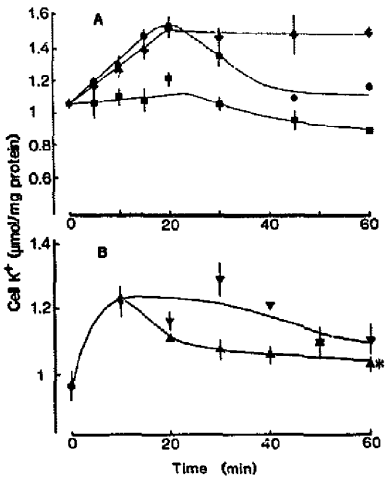

Fig. 7. Time-dependent changes in the K^+ contents of HeLa cells after medium replacement with a medium containing 80 mM K^+, 70 mM Na^+ and 100 μM ouabain with or without DIDS or the same medium but its Cl^- was substituted with NO_3^- or gluconate. (A) Addition of DIDS and substitution of medium Cl^- with gluconate. ●, control; ◆, 0.5 mM DIDS; ■, gluconate. (B) Substitution of medium Cl^- with NO_3^-. ▲, NO_3^-; ▼, NO_3^- and 1 mM DIDS. Points and bars are means and S.D. values for four samples. *, significantly different from value at time zero ($P < 0.01$).

Effects of DIDS and anion substitution

The cellular K^+ content increased and reached a maximum of 45% higher than the original level 20 min after medium replacement with the 80 mM K^+-medium and returned to the level at 45 min. Addition of an inhibitor of Cl^- channel DIDS (0.5 mM) strongly inhibited the regulatory decrease in the cellular K^+ content without giving influence to the initial rise of the content (Fig. 7A). When medium Cl^- was totally substituted with the same concentration of NO_3^-, the initial rise in the K^+ content occurred normally, but the regulatory K^+ decrease was partly inhibited, i.e., decreased to a level of 7% above the original level at 60 min (Fig. 7B). Addition of DIDS to the anion-substituted medium markedly but incompletely inhibited the regulatory decrease, the cellular K^+ content being 14% higher than the original value at 60 min. As a major portion of cellular Cl^- could be replaced with NO_3^- after 30 min (see Fig. 8), the incomplete inhibition of the regulatory K^+ decrease suggests that K^+ efflux may also be coupled with NO_3^- efflux through DIDS-insensitive anion channels. Total substitution of medium Cl^- with an impermeable anion gluconate strongly inhibited both the initial increase and the regulatory decrease in the K^+ content, which was reduced to a subnormal level of 15% lower than the original level in 60 min, suggesting cell shrinkage (Fig. 7A). The decrease in cellular K^+ would mainly be due to osmotically induced K^+ efflux towards Donnan equilibrium in the presence of the high concentration of the impermeable anion in the medium.

Change in the cellular Cl^- content

The cellular Cl^- content also showed the pattern of initial rise and delayed decrease after the medium replacement (Fig. 8). A maximum of 40% higher than the original level was attained at 10 min and returned to the level after 30 min. The pattern of change in

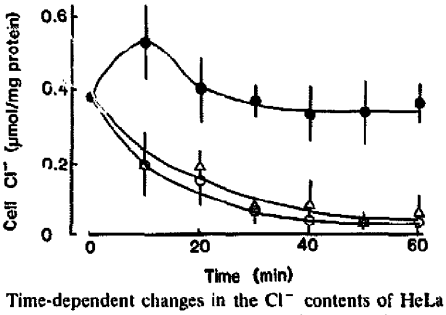

Fig. 8. Time-dependent changes in the Cl^- contents of HeLa cells after medium replacement with either a medium containing 80 mM K^+, 70 mM Na^+ and 100 μM ouabain or the same medium whose Cl^- was substituted with NO_3^-. The Cl^- contents were determined by preloading the cells with the isotope. ●, the 80 mM K^+-medium containing $^{36}Cl^-$; ○, the same medium without the isotope; △, the same medium without the isotope but NO_3^- substituted for medium Cl^-. Points and bars are means and S.D. values for four samples.

cellular Cl^- was similar to those observed for cellular K^+ (Fig. 2) and water (Fig. 4). Study on loss of Cl^- from the cells showed that the Cl^- content decreased exponentially with time after the medium replacement and came to an extremely low level in 60 min. Though this experiment and that shown in Fig. 3 were conducted separately, the pattern of Cl^- loss was similar to the time course of K^+ loss, except that the Cl^- loss started instantly after the medium change. However, the Cl^- content decreased to about a half of the original content in 10 min, which was not accompanied with K^+ efflux (see Fig. 3) and might be due to an unknown mechanism. Therefore, a decrease in cellular Cl^- of only about 0.2 μmol/mg protein after 10 min corresponded to about 0.8 μmol/mg protein for the decrease in the K^+ content. The discrepancy must be relevant not only to the difference of cellular pool sizes of both ions but also to faster turnover of Cl^- than that of K^+. Even when medium Cl^- was substituted with NO_3^-, Cl^- loss was not significantly influenced, indicating that cellular Cl^- was replaced with NO_3^-. More than half of cellular Cl^- seemed to be replaced with NO_3^- at 10 min and most of the Cl^- after 30 min. The difference of the values of normal cellular Cl^- content between those shown in Table I and Fig. 8 could arise from difference of methods used in the experiments. Only cellular Cl^- replaceable with medium Cl^- in 30 min was determined by the method used in the latter experiment.

Effects of other transport inhibitors

The effect of an addition of TEA (5 mM), furosemide (0.2 mM) or amiloride (1 mM) was tested. However, these inhibitors affected neither the initial increase, nor the regulatory decrease in the cellular K^+ content after the medium change (data not shown).

Discussion

Cell swelling has been observed when various cells are incubated in hyposmotic media as reviewed by Hoffmann and Simonsen [21]. The swelling is primarily due to water influx driven by osmotic force, which is accompanied by cation influx. Even in normal media containing Na^+ as a predominant cation, the osmotic force principally acts to drive water inward because of the presence of impermeant anions in cells [19]. However, transmembrane movement of water is osmotically restricted, since Na^+ is relatively impermeable; and so the cells do not swell. In fact, many types of cells including duck red cells [3], *Amphiuma* red cells [22] and HeLa cells do not swell in normal culture medium, irrespective of the presence of ouabain.

Then, if the isosmotic medium contains either a cation ionophore or a high concentration of a highly permeable substance, cell swelling could occur. It has been reported that membrane permeability of Na^+ increases by addition of nystatin or monensin and, hence, the cellular Na^+ content increases and the K^+ content decreases toward Donnan equilibrium [23,24]. Because of the Na^+ accumulation, the sum of the Na^+ and K^+ contents of BHK cells has been proved to increase in the presence of monensin, which osmotically induces cell swelling [25]. Similar swelling of HeLa cells caused by nystatin can be prevented by adding 60 mM sucrose to the medium [19]. Swelling in isosmotic media has been shown for toad bladder cells in the presence of high concentrations of urea [26]. Similar cell swelling occurs in isosmotic media when medium Na^+ is replaced wholly or in part by K^+ [27,28].

In the present study, we reported changes in the cation contents of HeLa cells upon replacing normal medium to isosmotic high-K^+ and low-Na^+ media. Results showed an increase in the K^+ content per cell or per mg of cell protein depending on the medium K^+ concentration. When the medium was limited to that containing 70 mM Na^+ and 80 mM K^+ (80 mM K^+-medium), net increases in both water, K^+ and Cl^- contents were initially demonstrated. The net K^+ gain was not inhibited by various agents including quinine, BAPTA-AM, DIDS, TEA, furosemide and amiloride. Only replacement of medium Cl^- with an impermeable anion gluconate markedly suppressed the K^+ gain. These results reveal that the K^+ uptake from the isosmotic high K^+-media is a phenomenon associated with inward water movement osmotically driven toward Donnan equilibrium.

HeLa cells have been reported not to show RVD in hyposmotic medium, but they swell to smaller extents than those expected when they are assumed to behave as ideal osmometers [2]. The weak swelling could relate to a swelling-induced K^+ loss. Such a K^+ loss seemed also to occur in the isosmotic high K^+-media used in the present study. After attaining a supranormal peak of the cellular K^+ content upon transferring the cells to the 80 mM K^+-medium, K^+ loss exceeded its uptake, accompanied by a decrease in cell water, i.e., RVD. The K^+ loss was completely inhibited by addition of 1 mM quinine or 10 μM BAPTA and partly by that of trifluoperazine or removal of medium Ca^{2+}. These inhibitory effects suggest that the K^+ efflux takes place mainly through Ca^{2+}-dependent K^+ channels. A similar role of the K^+ channels in RVD has been reported for Ehrlich ascites tumor cells [10], *Amphiuma* red cells [9], human lymphocytes [13] and cultured renal cells [29], placed in hyposmotic media and toad bladder cells in isosmotic media containing high concentrations of a highly permeant substance urea [26]. The regulatory changes of cellular K^+ and

water observed in the present study occurred at much slower rates than those of the reported RVD.

An increase in the cellular Ca^{2+} concentration associated with hyposmotic cell swelling has been reported for *Amphiuma* red cells [9], toad bladder cells [26], and cultured intestinal cells [30]. In addition to the swelling-induced change, an increase in cellular Ca^{2+} due to activation of Ca^{2+} channels by membrane depolarization has been reported for mouse neuroblastoma cells [31] and rat PC12 cells [32]. Stimulation of intracellular Ca^{2+} release by membrane depolarization is also indicated in cardiac myocytes [33]. Membrane potential of HeLa cells changes from about −38 mV in the normal medium to about −22 mV in the 80 mM K^+-medium (data not shown). Therefore, the increase in cellular Ca^{2+} after the medium replacement may be caused by membrane depolarization. In the present study, we showed instant and transient increase in cellular Ca^{2+} after the medium change. The increase could be a trigger for opening Ca^{2+}-dependent K^+ channels. However, the opening of the K^+ channels may be delayed for a certain lag time following the increase in cellular Ca^{2+} in HeLa cells. The reason for this is not known, however, but such an implication was supported by the insignificant effect of ionomycin on the regulatory K^+ decrease, despite that the addition of ionophore must increase cellular Ca^{2+} instantly.

Transmembrane movement of a cation is coupled with parallel movement of anion(s). The coupling is essential for maintaining electroneutrality and for the cation movement steadily to continue. The initial increase in the cellular K^+ content after medium replacement to the 80 mM K^+-medium was unaffected by substitution of medium Cl^- with a permeable anion NO_3^-. This reveals that inward movement of K^+ can be supported not only by Cl^- but also by NO_3^- as a counter anion for K^+.

In contrast to the initial increase in cellular K^+, its regulatory decrease was suppressed strongly by addition of DIDS and partly by NO_3^--substitution for medium Cl^-. In the latter case, a major part of cellular Cl^- proved to be replaced with NO_3^- during the time course of regulatory decrease (Fig. 8). If we assume that K^+ efflux is coupled only with Cl^- efflux but not with NO_3^- efflux, then the K^+ efflux would be reduced due to the decrease in cellular Cl^-. Addition of DIDS must completely inhibit it, since perfect inhibition of the regulatory K^+ decrease by the agent was demonstrated when cellular anion was Cl^- (Fig. 7A). Though the K^+ decrease was suppressed in the NO_3^--substituted medium and addition of DIDS further inhibited it (Fig. 7B), this assumption is unlikely. The reason is that the addition of DIDS did not perfectly inhibit the K^+ decrease in the NO_3^--substituted medium. Thus the regulatory K^+ decrease would mainly be associated with NO_3^- efflux in the NO_3^--substituted medium, which would be less sensitive to DIDS than Cl^- efflux. This suggests the use of other pathway(s) in addition to DIDS-sensitive anion channels.

Our results explain that, when the high K^+-medium contains Cl^-, the regulatory K^+ decrease takes place through Ca^{2+}-dependent K^+ channels and is supported by Cl^- efflux through DIDS-sensitive anion channels. In the NO_3^--substituted medium, K^+ efflux is coupled with NO_3^- efflux mediated by both DIDS-sensitive and -insensitive channels, though K^+ efflux in total is reduced compared to that in the Cl^--containing medium. This implies that the K^+ efflux is more selectively associated with Cl^- efflux than efflux of NO_3^- or probably another anion. Importance of the Cl^--selective channels for RVD in hyposmotic media has been reported for various types of cells [10,11,29].

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